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(54) Title: CLONING AND PRODUCTION OF HUMAN VON WILLEBRAND FACTOR GPIb BINDING DOMAIN POLYPEPTIDES AND METHODS OF USING SAME

(57) Abstract

The subject invention provides non-glycosylated, biologically active polypeptides which comprise the vWF (von Willebrand Factor) GPIb binding domain. These polypeptides may be used to inhibit platelet adhesion and aggregation in the treatment of subjects with conditions such as cerebrovascular disorders and cardiovascular disorders. This invention also provides expression plasmids encoding these polypeptides as well as methods of producing by transforming a bacterial cell and recovering such polypeptides. In addition, the subject invention provides methods of treating and preventing cerebrovascular, cardiovascular and other disorders using these polypeptides to inhibit platelet aggregation.

CLONING AND PRODUCTION OF HUMAN VON WILLEBRAND FACTOR GPIb BINDING DOMAIN POLYPEPTIDES AND METHODS OF USING SAME

Background of the Invention

This application is a continuation-in-part of U.S. Serial No. 487,767, filed March 2, 1990, the contents of which are hereby incorporated by reference into the present disclosure.

Throughout this application various publications are referenced within parentheses. The disclosures of these publications in their entireties are hereby incorporated by reference in this application in order to more fully describe the state of the art to which this invention pertains.

This invention relates to the cloning and production of human von Willebrand Factor analogs and methods of using such analogs.

Structural Features of von Willebrand Factor

Von Willebrand Factor (vWF) is a large plasma protein which is synthesized in the endothelial cells which form the inner surface lining of the blood vessel wall, and by megakarocytes, the precursor of platelets. Large amounts of vWF are found in platelet α-granules, whose contents are released into the blood upon platelet activation. Newly synthesized vWF in endothelial cells may enter the blood via two alternative pathways. Part is secreted constitutively into the blood, mainly as disulfide-linked dimers or small multimers of a 250,000 dalton subunit. Alternatively, part enters secretory organelles called Weibel-Palade bodies. The vWF stored in Weibel-Palade bodies is highly multimeric, ranging in size from that of a dimer to multimers of 50 or

more subunits, and can be released from the cells by treatment with secretatogues, such as thrombin. The highly multimeric vWF is the most effective in promoting platelet adhesion.

5   The gene encoding vWF has been isolated and shown to be greater than 150 kb in length. It is composed of over 20 exons. The vWF mRNA is approximately 9000 bases in length and encodes a pre-pro-vWF of 2813 amino acids. Residues 1-
10   22 form a processed leader sequence which presumably is cleaved after entry of the protein into the rough endoplasmic reticulum. The N-terminal portion of the pro-vWF (741 amino acids) is the pro-peptide which is not present in mature vWF. This peptide is present in the blood
15   and has been shown to be identical to a blood protein previously known as von Willebrand Antigen II (vW AgII). The pro-peptide is essential for the multimerization of vWF. Cells into which a vWF cDNA containing only mature vWF sequences have been introduced produce only dimers. No
20   function is known for the propeptide/vW AgII.

DNA sequence analysis has demonstrated that the pro-vWF precursor is composed of repeated domain subunits. Four different domains have been identified. Mature vWF consists
25   of three A type, three B type, and two C type domains. There are also two complete and one partial D type domain. The pro-peptide consists of two D type domains, leading to the speculation that it may have associated functions.

30   Mature vWF is a multivalent molecule which has binding sites for several proteins. One of the binding sites recognizes the platelet glycoprotein Ib (GPIb). Using proteolytic digests this site has been localized to the region between amino acid residues 449 and 728 of mature vWF. In addition,
35   vWF has at least two collagen binding sites, at least two

heparin binding sites, a Factor VIII binding site, and a RGD
site which binds to the platelet GP IIb/IIIa receptor.

Involvement Of vWF In Platelet Adhesion To Subendothelium

Evidence that vWF, and specifically, the binding of vWF to
the platelet GPIb receptor, is essential for normal platelet
adhesion, is based on both clinical observations and in
vitro studies. Patients with the bleeding disorder von
Willebrand Disease (vWD) have reduced levels of vWF or are
completely lacking in vWF. Alternatively, they may have
defective vWF. Another disorder, Bernard-Soulier Syndrome
(BSS), is characterized by platelets lacking GPIb receptors.

The in vitro system which most closely approximates the
environment of a damaged blood vessel consists of a
perfusion chamber in which an everted blood vessel segment
(rabbit aorta, human post-mortem renal artery, or the human
umbilical artery) is exposed to flowing blood. Aft r
stripping off the layer of endothelial cells from the
vessel, blood is allowed to flow through the chamber. The
extent of platelet adhesion is estimated directly by
morphometry or indirectly using radiolabeled platelets.
Blood from patients with VWD or BSS does not support
platelet adhesion in this system while normal blood does,
indicating the need for vWF and platelet GPIb. Moreover,
addition of monoclonal antibodies to GPIb prevents platelet
adhesion as well. The vWF-dependence of platelet adhesion
is more pronounced under conditions of high shear rates,
such as that present in arterial flow. Under conditions of
low shear rates, platelet adhesion may be facilitated by
other adhesion proteins, such as fibronectin. Possibly, the
adhesive forces provided by these other prot ins are not
adequate to support adhesion at high shear forc s, and vWF
dependence becomes apparent. Also, the multimeric natur of

the vWF may provide for a stronger bond by binding more sites on the platelet.

About 20% of patients from whom clots have been removed by angioplasty or by administration of tissue plasminogen activator (tPA) suffer re-occlusion. This is presumably the result of damage to the endothelium during the treatment which results in the adhesion of platelets to the affected region on the inner surface of the vessel. This is followed by the aggregation of many layers of platelets and fibrin onto the previously adhered platelets, forming a thrombus.

To date none of the anti-platelet aggregation agents described in the literature prevent the initial platel t adhesion to the exposed sub-endothelium thereby preventing subsequent clot formation.

The subject invention provides non-glycosylated, biologically active polypeptides which comprise the vWF (von Willebrand Factor) GP1b binding domain. These polypeptides may be used inter alia to inhibit platelet adhesion and aggregation in the treatment of subjects with conditions such as cerebrovascular disorders and cardiovascular disorders. This invention also provides expression plasmids encoding these polypeptides as well as methods of producing by transforming a bacterial cell and recovering such polypeptides. In addition, the subject invention provides methods of treating and preventing cerebrovascular, cardiovascular and other disorders using these polypeptides to inhibit platelet aggregation.

Summary of the Invention

This invention provides a non-glycosylated, biologically active polypeptide having the amino acid sequence:

X-A-[Cys Ser Arg Leu Leu Asp Leu Val Phe Leu Leu Asp Gly
Ser Ser Arg Leu Ser Glu Ala Glu Phe Glu Val Leu Lys Ala
Phe Val Val Asp Met Met Glu Arg Leu Arg Ile Ser Gln Lys
Trp Val Arg Val Ala Val Val Glu Tyr His Asp Gly Ser His
Ala Tyr Ile Gly Leu Lys Asp Arg Lys Arg Pro Ser Glu Leu
Arg Arg Ile Ala Ser Gln Val Lys Tyr Ala Gly Ser Gln Val
Ala Ser Thr Ser Glu Val Leu Lys Tyr Thr Leu Phe Gln Ile
Phe Ser Lys Ile Asp Arg Pro Glu Ala Ser Arg Ile Ala Leu
Leu Leu Met Ala Ser Gln Glu Pro Gln Arg Met Ser Arg Asn
Phe Val Arg Tyr Val Gln Gly Leu Lys Lys Lys Lys Val Ile
Val Ile Pro Val Gly Ile Gly Pro His Ala Asn Leu Lys Gln
Ile Arg Leu Ile Glu Lys Gln Ala Pro Glu Asn Lys Ala Phe
Val Leu Ser Ser Val Asp Glu Leu Glu Gln Gln Arg Asp Glu
Ile Val Ser Tyr Leu Cys]-B-COOH

wherein X is NH_2-methionine- or NH_2-;

A is a sequence of at least 1, but less than 35 amino acids, which sequence is present in naturally occurring vWF, the carboxy terminal amino acid of which is the tyrosine #508 shown in Figure 12;

B is a sequence of at least 1, but less than 211 amino acids, which sequence is present in naturally occurring vWF, the amino terminal amino acid of which is the aspartic acid #696 shown in Figure 12; and

the two cysteines included within the bracketed sequence are joined by a disulfide bond.

In addition, the subject invention provides a method of
producing any of the above-described polypeptides which
comprises transforming a bacterial cell with an expression
plasmid encoding the polypeptide, culturing the resulting
5   bacterial cell so that the cell produces the polypeptide
encoded by the plasmid, and recovering the polypeptide so
produced.

Furthermore, the subject invention provides a pharmaceutical
10   composition comprising an amount of any of the above-
described polypeptides effective to inhibit platelet
aggregation and a pharmaceutically acceptable carrier. The
subject invention also provides a method of inhibiting
platelet aggregation which comprises contacting platelets
15   with an amount of any of the above-described polypeptides
effective to inhibit platelet aggregation. In addition, the
subject invention provides methods of treating, preventing
or inhibiting disorders such as cerebrovascular or
cardiovascular disorders or thrombosis, comprising
20   administering to the subject an amount of any of the above-
described polypeptides effective to treat or prevent such
disorders.

The subject invention also provides a method for recovering
25   a purified, biologically active above-described polypeptide
which comprises:

(a) producing in a bacterial cell a first
polypeptide having the amino acid
30   sequence of the polypeptide but lacking
the disulfide bond;

(b) disrupting the bacterial cell so as to
produce a lysate containing the first
35   polypeptide;

(c) treating the lysate so as to obtain inclusion bodies containing the first polypeptide;

5

(d) contacting the inclusion bodies from step (c) so as to obtain the first polypeptide in soluble form;

10

(e) treating the resulting first polypeptide so as to form the biologically active polypeptide;

(f) recovering the biologically active polypeptide so formed; and

15

(g) purifying the biologically active polypeptide so recovered.

Bri f Descripti n of the Figur s

Figure 1: Construction of pvW1P

5   This figure shows the construction of plasmid pvW1P. A
       series of vWF cDNA clones in λ gt11 (isolated from a human
       endothelial cell cDNA library) were isolated.   One cDNA
       clone covering the entire GPIb binding domain was subcloned
       into the EcoRI site of pUC19. The resulting plasmid, pvW1P,
10   contains a 2.5 kb cDNA insert.

Figure 2: Construction of pvWF-VA1

       This figure shows the construction of plasmid pvWF-VA1. A
15   synthetic oligomer containing an ATG initiation codon
       located before the amino acid glu-437 (i.e., the 437th amino
       acid in the vWF protein shown in Figure 12) was ligated to
       plasmid pvW1P digested with NdeI and Bsu36I. The resulting
       plasmid was designated pvWF-VA1, and has been deposited in
20   E. coli strain Sφ930 under ATCC Accession No. 68530.

Figure 3: Construction of pvWF-VB1

       This figure shows the construction of plasmid pvWF-VB1. A
25   synthetic oligomer containing an ATG initiation codon
       located before the amino acid phe-443 (see Figure 12) was
       ligated to plasmid pvW1p digested with NdeI and Bsu36I. The
       resulting plasmid was designated pvWF-VB1.

30   ### Figure 4: Construction of pvWF-VA2

       This figure shows the construction of plasmid pvWF-VA2. A
       synthetic oligomer containing a TAA termination codon
       located after the amino acid lys-728 (see Figure 12) was
35   ligated to plasmid pvWF-VA1 digested with HindIII and XmaI.

The resulting plasmid was designated pvWF-VA2.

Figure 5:   Construction of pvWF-VB2

5   This figure shows the construction of plasmid pvWF-VB2. A
synthetic oligomer containing a TAA termination codon was
ligated to plasmid pvWF-VB1 digested with HindIII and XmaI.
The resulting plasmid was designated pvWF-VB2.

10   **Figure 6**:   Construction of pvWF-VA3

This figure shows the construction of plasmid pvWF-VA3. An
NdeI-EcoRV fragment was isolated from plasmid pvWF-VA2 and
ligated to plasmid pMF-945 (constructed as described in
15   Figure 11) digested with NdeI and PvuII. The plasmid
obtained was designated pvWF-VA3. The plasmid expresses VA,
a vWF GPIb binding domain polypeptide which includes amino
acids 437 to 728 (see Figure 12) under the control of the
deo P_1P_2 promoter.

20

Figure 7:   Construction of pvWF-VB3

This figure shows the construction of plasmid pvWF-VB3. An
NdeI-EcoRV fragment was isolated from plasmid pvWF-VB2 and
25   ligated to plasmid pMF-945 digested with NdeI and PvuII.
The plasmid obtained was designated pvWF-VB3. The plasmid
expresses VB, a vWF GPIb binding domain polypeptide which
includes amino acids 443 to 728 under the control of the deo
P_1P_2 promoter.

30

Figure 8:   Construction of pvWF-VC3

This figure shows the construction of plasmid pvWF-VC3. A
synthetic linker was ligated to pvWF-VA3 digested with NdeI
35   and Tth111I. The plasmid obtained was designated pvWF-VC3,

and has been deposited with the ATCC under ATCC Accession
No. 68241. The plasmid expresses VC (also referred to as
VCL or VC3), a vWF GPIb binding domain polypeptide which
includes amino acids 504 to 728 (see Figure 12) under the
5   control of the deo P_1P_2 promoter.

Figure 9: Construction of pvWF-VD3

This figure shows the construction of plasmid pvWF-VD3. A
10 synthetic linker was ligated to pvWF-VA3 digested with NdeI
and Tth111I. The plasmid obtained was designated pvWF-VD3.
The plasmid expresses VD, a vWF GPIb binding domain poly-
peptide which includes amino acids 513 to 728 (see Figure
12) under the control of the deo P_1P_2 promoter.

15

Figure 10: Relative Alignment of Plasmids Expressing vWF-
GPIb Binding Domain Polypeptides

This figure shows the relative alignment of the plasmids
20 expressing the vWF-GPIb binding domain polypeptides. Also
shown on the top two lines are representations of the vWF
cDNA and the location of the GPIb binding domain coding
region within the cDNA.

25 **Figure 11**: Construction of Plasmid pMF-945

This figure shows the construction of plasmid pMF-945.
Plasmid pEFF-920 (in Escherichia coli Sϕ930, ATCC Accession
No. 67706) was cleaved with BglII and NdeI, and the large
30 fragment was isolated. This fragment was ligated to the
small 540 bp fragment produced by cleaving plasmid pMF-5534
(ATCC Accession No. 67703) with BglII and NdeI. This
produces plasmid pMF-945 which harbors the PAR sequence and
in 5' and 3' order the deo P_1P_2 promoter sequences, the
35 modified deo ribosomal binding site with an enhancer

sequence, a pGH analog coding sequence and the T_1T_2 transcription termination sequenc s. Plasmid pMF-945 is a high level expressor of pGH analog protein.

Figure 12: **Translated cDNA Sequence of Mature Human vWF**

This figure which consists of Figures 12A, 12B, 12C, 12D, 12E, 12F, 12G and 12H shows the translated cDNA sequence of mature human von Willebrand Factor.

This sequence was compiled using the data disclosed by Verweij, C.L., et al., EMBO Journal 5: 1839-1847 (1986) and Sadler, J.E., et al., Proc. Natl. Acad. Sci. 82: 6394-6398 (1985). This nucleotide sequence commences with nucleotide number 2519 (where nucleotide 1 relates to the start of the coding sequence for the signal peptide) and terminates with nucleotide 8668, a total of 6150 nucleotides encoding mature vWF consisting of 2050 amino acids. The translated amino acid sequence commences with amino acid number 1 and terminates with amino acid number 2050. The corresponding nucleotide and amino acid designations are used throughout this application.

Figure 13: **Translated Sequence of VC, the vWF GPIb Binding Domain Polypeptide Expressed by Plasmids pvWF-VC3 and pvWF-VCL**

This figure shows the translated sequence of the von Willebrand Factor GPIb binding domain polypeptide expressed by plasmids pvWF-VC3 (ATCC Accession No. 68241) and pvWF-VCL (ATCC Accession No. 68242).

The first codon ATG encoding the translation initiation codon methionine has been added to the nucleotide sequence corresponding to nucleotides 4028 to 4702 of the sequence of

Figure 12. This sequence encodes a polypeptide containing
225 amino acids (plus the initiation methionine)
corresponding to amino acid Leu 504 to amino acid Lys 728 of
Figure 12, i.e. 226 amino acids in total.

5

Figure 14: Construction of pvWF-VCL

This figure shows the construction of plasmid pvWF-VCL.
Plasmid pvWF-VC3 was digested with HindIII and StyI and the
10   860 base pair fragment isolated. This fragment was ligated
with the large fragment isolated from the HindIII-StyI
digest of plasmid pMLK-100. The resulting plasmid was
designated pvWF-VCL and deposited in E.coli 4300(F⁻) with
the ATCC under ATCC Accession No. 68242. This plasmid
15   expresses VCL, the same vWF GPIb binding domain polypeptide
as pvWF-VC3 (methionine plus amino acids 504-728), however
under control of the λP_L promoter and the deo ribosomal
binding site.

20   **Figure 15**: Construction of Plasmid pvWF-VE2

Plasmid pvWF-VA2 was digested with NdeI and PstI and the
large fragment isolated. Synthetic oligomers No. 2 and No.
3 (shown in Figure 16) were treated with T4 polynucleotide
25   kinase. The large pvWF-VA2 fragment was then ligated with
synthetic oligomers No. 1 and No. 4, (shown in Figure 16)
and with kinased oligomers No. 2 and No. 3. The resulting
plasmid was designated pvWF-VE2.

30   **Figure 16**: Synthetic Oligomers Used in Construction of
pvWF-VE2.

This figure shows the four synthetic linkers (Nos. 1-4) used
in construction of pvWF-VE2.

35

Figur 17: <u>Construction of Plasmid pvWF-VE3</u>

Plasmid pvWF-VE2 was digested with NdeI and HindIII and the small 770 bp fragment isolated and ligated with the large fragment isolated from the NdeI-HindIII digest of plasmid pMLK-7891. The resulting plasmid was designated pvWF-VE3.

Figure 18: <u>Construction of Plasmid pvWF-VEL</u>

Plasmid pvWF-VE3 was digested with XmnI, treated with bacterial alkaline phosphatase (BAP), and further digested with NdeI and HindIII. Plasmid pMLK-100 was digested with NdeI and HindIII and treated with BAP. The two digests were mixed and ligated, producing plasmid pvWF-VEL which expresses the DNA sequence corresponding to amino acids 469-728 of mature vWF under the control of the λP_L promoter and the cII ribosomal binding site.

Figure 19:   <u>The Effect of VCL on BJV-Induced Aggregation in Human Platelet Rich Plasma (PRP)</u>
This figure provides the results of a standardized von Willebrand Factor (vWF)-dependent aggregation assay using human PRP.

Figure 20:   <u>The Effect of VCL on BJV-Induced Aggregation in Rat PRP</u>
This figure provides the results of a standardized von Willebrand Factor (vWF)-dependent aggregation assay using rat PRP.

Detailed Description of the Invention

The plasmids pvWF-VC3, pvWF-VCL and pvWF-VA1 were deposited
in Escherichia coli pursuant to, and in satisfaction of, the
5   requirements of the Budapest Treaty on the International
Recognition of the Deposit of Microorganisms for the
Purposes of Patent Procedure with the American Type Culture
Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland
20852 under ATCC Accession Nos. 68241, 68242 and 68530,
10   respectively.

This invention provides a non-glycosylated, biologically
active polypeptide having the amino acid sequence:

15   X-A-[Cys Ser Arg Leu Leu Asp Leu Val Phe Leu Leu Asp Gly
          Ser Ser Arg Leu Ser Glu Ala Glu Phe Glu Val Leu Lys Ala
          Phe Val Val Asp Met Met Glu Arg Leu Arg Ile Ser Gln Lys
          Trp Val Arg Val Ala Val Val Glu Tyr His Asp Gly Ser His
          Ala Tyr Ile Gly Leu Lys Asp Arg Lys Arg Pro Ser Glu Leu
20   Arg Arg Ile Ala Ser Gln Val Lys Tyr Ala Gly Ser Gln Val
          Ala Ser Thr Ser Glu Val Leu Lys Tyr Thr Leu Phe Gln Ile
          Phe Ser Lys Ile Asp Arg Pro Glu Ala Ser Arg Ile Ala Leu
          Leu Leu Met Ala Ser Gln Glu Pro Gln Arg Met Ser Arg Asn
          Phe Val Arg Tyr Val Gln Gly Leu Lys Lys Lys Lys Val Ile
25   Val Ile Pro Val Gly Ile Gly Pro His Ala Asn Leu Lys Gln
          Ile Arg Leu Ile Glu Lys Gln Ala Pro Glu Asn Lys Ala Phe
          Val Leu Ser Ser Val Asp Glu Leu Glu Gln Gln Arg Asp Glu
          Ile Val Ser Tyr Leu Cys]-B-COOH

30   wherein X is NH$_2$-methionine- or NH$_2$-;

          A is a sequence of at least 1, but less than 35 amino
          acids, which sequence is present in naturally occurring
          human vWF, the carboxy terminal amino acid of which is
35   the tyrosine #508 shown in Figure 12;

B is a sequence of at least 1, but less than 211 amino
acids, which sequence is present in naturally occurring
human vWF, the amino terminal amino acid of which is the
aspartic acid #696 shown in Figure 12; and

5

the two cysteines included within the bracketed sequence
are joined by a disulfide bond. The bracketed sequence
comprises amino acids #509-#695 of Figure 12.

10   In one embodiment, this polypeptide has the amino acid
sequence:

    X-[Leu His Asp Phe Tyr Cys Ser Arg Leu Leu Asp Leu Val
    Phe Leu Leu Asp Gly Ser Ser Arg Leu Ser Glu Ala Glu Phe
    Glu Val Leu Lys Ala Phe Val Val Asp Met Met Glu Arg Leu
15 Arg Ile Ser Gln Lys Trp Val Arg Val Ala Val Val Glu Tyr
    His Asp Gly Ser His Ala Tyr Ile Gly Leu Lys Asp Arg Lys
    Arg Pro Ser Glu Leu Arg Arg Ile Ala Ser Gln Val Lys Tyr
    Ala Gly Ser Gln Val Ala Ser Thr Ser Glu Val Leu Lys Tyr
    Thr Leu Phe Gln Ile Phe Ser Lys Ile Asp Arg Pro Glu Ala
20 Ser Arg Ile Ala Leu Leu Leu Met Ala Ser Gln Glu Pro Gln
    Arg Met Ser Arg Asn Phe Val Arg Tyr Val Gln Gly Leu Lys
    Lys Lys Lys Val Ile Val Ile Pro Val Gly Ile Gly Pro His
    Ala Asn Leu Lys Gln Ile Arg Leu Ile Glu Lys Gln Ala Pro
    Glu Asn Lys Ala Phe Val Leu Ser Ser Val Asp Glu Leu Glu
25 Gln Gln Arg Asp Glu Ile Val Ser Tyr Leu Cys Asp Leu Ala
    Pro Glu Ala Pro Pro Pro Thr Leu Pro Pro Asp Met Ala Gln
    Val Thr Val Gly Pro Gly Leu Leu Gly Val Ser Thr Leu Gly
    Pro Lys]-COOH

30   wherein X is NH$_2$- or NH$_2$-methionine-, preferably
    NH$_2$-methionine-.

The bracketed sequence comprises amino acids #504-#728 of
Figure 12.

35

-16-

One skilled in the art to which the subject invention pertains can readily make such polypeptides using recombinant or non-recombinant DNA techniques.

5   The polypeptides may be constructed using recombinant DNA technology. One means for obtaining the polypeptides is to express nucleic acid encoding the polypeptides in a suitable host, such as a bacterial, yeast, or mammalian cell, using methods well known in the art, and recovering the

10 polypeptide after it has been expressed in such a host.

Examples of vectors that may be used to express the nucleic acid encoding the polypeptides are viruses such as bacteriophages (such as phage lambda), cosmids, plasmids,

15 and other recombination vectors. Nucleic acid molecules are inserted into vector genomes by methods well known in the art. For example, using conventional restriction enzyme sites, insert and vector DNA can both be exposed to a restriction enzyme to create complementary ends on both

20 molecules which base pair with each other and are then ligated together with a ligase. Alternatively, linkers can be ligated to the insert DNA which correspond to a restriction site in the vector DNA, which is then digested with the restriction enzyme which cuts at that site. Other

25 means are also available.

Vectors comprising nucleic acid encoding the polypeptides may be adapted for expression in a bacterial cell, a yeast cell, or a mammalian cell which additionally comprise the

30 regulatory elements necessary for expression of the nucleic acid in the bacterial, yeast, or mammalian cells so located relative to the nucleic acid encoding the polypeptide as to permit expression thereof. Regulatory elements required for expression include promoter sequences to bind RNA polymerase

35 and transcription initiation sequences for ribosome binding.

For example, a bacterial expression vector may include a promoter such as the λ P_L or deo promoters and for transcription initiation the C_{II} or deo ribosomal binding sites. Such vectors may be obtained commercially or
5   assembled from the sequences described by methods well known in the art, for example the methods described above for constructing vectors in general.

In addition, non-recombinant techniques such as chemical
10   synthesis, synthetic DNA or cDNA may be used to obtain the above-described polypeptides. One means of isolating the polypeptide is to probe a human genomic library with a natural or artificially designed DNA probe, using methods well known in the art. DNA and cDNA molecules which encode
15   the polypeptides may be used to obtain complementary genomic DNA, cDNA or RNA from human, mammalian or other animal sources, or to isolate related cDNA or genomic clones by the screening of cDNA or genomic libraries.

20   The subject invention further provides a pharmaceutical composition comprising an amount of any of the above-described polypeptides effective to inhibit platelet aggregation and a pharmaceutically acceptable carrier.

25   As used herein, the term "pharmaceutically acceptabl carrier" encompasses any of the standard pharmaceutical carriers. Such carriers are well known in the art and may include, but are in no way and are not intended to be limited to, any of the standard pharmaceutical carriers such
30   as phosphate buffered saline solutions, water, emulsions such as oil/water emulsion, and various types of wetting agents. Other carriers may also include sterile solutions, tablets, coated tablets, and capsules.

35   Typically such carriers contain excipients such as starch,

milk, sugar, certain types of clay, gelatin, stearic acid or
salts thereof, magnesium or calcium stearate, talc,
vegetable fats or oils, gums, glycols, or other known
excipients. Such carriers may also include flavor and color
5   additives or other ingredients. Compositions comprising
such carriers are formulated by well known conventional
methods.

The composition has an amount sufficient to result in a
10   blood concentration of 0.06 to 58 μM, preferably between
about 0.06 and 29 μM, for example 0.23 to 23 μM. Expressed
in different terms, the amount should be 0.1 to 100 mg/Kg
body weight, preferably 0.1 to 50 mg/Kg body weight, for
example 0.4 to 40 mg/kG body weight.

15

The administration of the composition may be effected by any
of the well known methods, including but not limited to
intravenous, intramuscular, subcutaneous and oral
administration.

20

This invention also provides a method of inhibiting platelet
aggregation which comprises contacting platelets with an
amount of any of the above-described polypeptides effective
to inhibit platelet aggregation so as to inhibit platelet
25   aggregation.

This invention also provides expression plasmids encoding
the above-described polypeptides. In one embodiment, the
expression plasmid encoding the polypeptide with the brack-
30   eted sequence, i.e. amino acids #504-#728 of Figure 12, is
designated pvWF-VC3 and is deposited under ATCC Accession
No. 68241. In another embodiment, the expression plasmid
encoding a polypeptide with the bracketed sequence, i.e.
amino acids #504-#728 of Figure 12, is designated pvWF-VCL
35   and is deposited under ATCC Accession No. 68242.

The expression plasmids of this invention further comprise
suitable regulatory elements positioned within the plasmid
relative to the DNA encoding the polypeptide so as to effect
expression of the polypeptide in a suitable host cell, such
as promoter and operators, e.g. deo P_1P_2 and λ P_LO_L,
ribosomal binding sites, e.g. deo and C_{II}, and repressors.
Other suitable regulatory elements include, for example, the
lac, trp, tac, and lpp promoters (European Patent
Application Publication No. 0303972, published February 22,
1989).

The suitable regulatory elements are positioned within the
plasmid relative to the DNA encoding the polypeptide so as
to effect expression of the polypeptide in a suitable host
cell.   In preferred embodiments of the invention, the
regulatory elements are positioned close to and upstream of
the DNA encoding the polypeptide.

The expression plasmids of this invention may be introduced
into suitable host cells, preferably bacterial host cells.
Preferred bacterial host cells are Escherichia coli cells.
Examples of suitable Escherichia coli cells are strains
Sϕ930 or 4300, but other Escherichia coli strains and other
bacteria can also be used as host cells for the plasmids.
Such bacteria include Pseudomonas aeruginosa and Bacillus
subtilis.

The bacteria used as hosts may be any strain including
auxotrophic (such as A1645), prototrophic (such as A4255),
and lytic strains; F^+ and F^- strains; strains harboring the
cI^{857} repressor sequence of the λ prophage (such as A1645
and A4255); and strains deleted for the deo repressors and
the deo gene (see European Patent Application Publication
No. 0303972, published February 22, 1989). Escherichia coli
strain A4255 (F^+) has been deposited under ATCC Accession

No. 53468, and <u>Escherichia coli</u> strain A1645 has been deposited under ATCC Accession No. 67829.

5   The invention provides a bacterial cell which comprises these expression plasmids. In one embodiment, the bacterial cell is an <u>Escherichia coli</u> cell. In preferred embodiments, the invention provides an <u>Escherichia coli</u> cell containing the plasmid designated pvWF-VA1, deposited in <u>E. coli</u> strain Sφ930 with the ATCC under ATCC Accession No. 68530; pvWF-

10   VA3; pvWF-VB3; pvWF-VC3, deposited in <u>E. coli</u> strain Sφ930 with the ATCC under ATCC Accession No. 68241; pvWF-VD3; or pvWF-VCL, deposited in <u>E. coli</u> strain 4300(F⁻) with the ATCC under ATCC Accession No. 68242.

15   All the <u>E. coli</u> host strains described above can be "cured" of the plasmids they harbor by methods well-known in the art, e.g. the ethidium bromide method described by R.P. Novick in <u>Bacteriol. Review 33</u>, 210 (1969).

20   In addition, the subject invention provides a method of producing any of the above-described polypeptides which comprises transforming a bacterial cell with an expression plasmid encoding the polypeptide, culturing the resulting bacterial cell so that the cell produces the polypeptide

25   encoded by the plasmid, and recovering the polypeptide so produced.

Furthermore, the invention provides a method of treating a subject with a cerebrovascular disorder which comprises

30   administering to the subject an amount of any of the polypeptides of the invention effective to inhibit platelet aggregation.

Also provided is a method of treating a subject with a

35   cardiovascular disorder which comprises administering to the

subject an amount of a polypeptide effective to inhibit platelet aggregation. Examples of cardiovascular disorders susceptible to treatment include acute myocardial infarction or angina.

5

Further, the subject invention provides method of inhibiting platelet aggregation in a subject prior to, during, or after the subject has undergone angioplasty, thrombolytic treatment, or coronary bypass surgery which comprises

10   administering to the subject an amount of a polypeptide of the invention effective to inhibit platelet aggregation.

The invention also provides a method of maintaining blood vessel patency in a subject prior to, during, or after the

15   subject has undergone coronary bypass surgery, which comprises administering to the subject an amount of a polypeptide of the invention effective to inhibit platelet aggregation.

20   The invention also provides a method of treating a subject having cancer which comprises administering to the subject an amount of a polypeptide of the invention effective to retard tumor metastasis.

25   The invention also provides a method of inhibiting thrombosis in a subject which comprises administering to the subject an amount of a polypeptide of the invention effective to inhibit the thrombosis. The thrombosis may be associated with an inflammatory response.

30

In addition, the subject invention provides a polypeptide of the invention bound to a solid matrix.

The invention also provides a method of treating a subj ct

35   suffering from platelet adhesion to damaged vascular

surfaces which comprises administering to the subject an amount of the polypeptide of the invention effective to inhibit platelet adhesion to damaged vascular surfaces.

5   The invention also provides a method of preventing platelet adhesion to a prosthetic material or device in a subject which comprises administering to the subject an amount of the polypeptide of the invention effective to prevent platelet adhesion to the material or device.

10

The invention also provides a method of inhibiting re-occlusion in a subject following angioplasty or thrombolysis which comprises administering to the subject an amount of the polypeptide of the invention effective to inhibit re-

15   occlusion.

The invention also provides a method of preventing vaso-occlusive crises in a subject suffering from sickle cell anemia which comprises administering to the subject an

20   amount of the polypeptide of the invention effective to prevent vaso-occlusive crises.

The invention also provides a method of preventing arteriosclerosis in a subject which comprises administering

25   to the subject an amount of the polypeptide of the invention effective to prevent arteriosclerosis.

The invention also provides a method of thrombolytic treatment of thrombi-containing, platelet-rich aggregates in

30   a subject which comprises administering to the subject an amount of the polypeptide of the invention effective to treat thrombi-containing, platelet-rich aggregates.

The invention also provides a method of preventing platelet

35   activation and thrombus formation due to high shear forces

in a subject suffering from stenosed or partially obstructed arteries which comprises administering to the subject an amount of the polypeptide of the invention effective to prevent platelet activation and thrombus formation.

The invention also provides a method of preventing thrombin-induced platelet activation in a subject which comprises administering to the subject an amount of the polypeptide of the invention effective to prevent thrombin-induced platelet activation.

The invention also provides a method of preventing stenosis as a result of smooth muscle proliferation following vascular injury in a subject which comprises administering to the subject an amount of the polypeptide of the invention effective to prevent stenosis.

The invention also provides a method for recovering a purified, biologically active polypeptide of the invention which comprises:

(a)   producing in a bacterial cell a first polypeptide having the amino acid sequence of the polypeptide but lacking the disulfide bond;

(b)   disrupting the bacterial cell so as to produce a lysate containing the first polypeptide;

(c)   treating the lysate so as to obtain inclusion bodies containing the first polypeptide;

(d)   contacting the inclusion bodies from step

(c)   so as to obtain the first polypeptide
in soluble form;

(e)   treating the resulting first polypeptide
so as to form the biologically active
polypeptide;

(f)   recovering   the   biologically   active
polypeptide so formed; and

(g)   purifying the biologically active poly-
peptide so recovered.

Step (e) may comprise contacting the polypeptide with a
thiol-containing compound and disulfide so as to refold and
reoxidize the polypeptide. Preferably, the thiol-containing
compound is glutathione, thioredoxin, ß-mercaptoethanol or
cysteine.

The contacting of step (d) may be effected in the presence
of a denaturant such as guanidine hydrochloride or urea.

The recovery of the polypeptide in step (f) may comprise
removing the denaturant by dialysis.

In step (g), the biologically active polypeptide may be
purified by cation exchange chromatography.

The first polypeptide may also be purified by cation
exchange chromatography after step (d).

The examples which follow are set forth to aid in understanding the invention but are not intended to, and should not be so construed as to, limit its scope in any way. The examples do not include detailed descriptions for conventional methods employed in the construction of vectors, the insertion of genes encoding polypeptides of interest into such vectors or the introduction of the resulting plasmids into bacterial hosts. Such methods are well-known to those skilled in the art and are described in numerous publications including Sambrook, Fritsch and Maniatis, Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory Press, USA, (1989).

EXAMPLES

All the references to map positions correspond to the identically numbered positions along the translated nucleotide sequence of mature human von Willebrand Factor shown in Figure 12.

Example 1:   Cloning and Expression of vWF GP1b Binding Domain Polypeptides

cDNA Cloning of Human vWF GP1b Binding Domain

A human endothelial cDNA library (obtained from CLONTECH Laboratories, Inc.) in λ gt11 was screened for human vWF positive sequences using two synthetic DNA probes. The probes were synthesized according to the published DNA sequence (Sadler et al., Proc. Nat. Acad. Sci. 82: 6394-8 (1985) and Verweij et al., EMBO J. 3: 1829-47 (1986)) of human vWF (flanking 5' end and 3' end of the vWF domain known to bind the GPIb receptor) (see Figure 12).

The synthetic probes have the following sequences:

Sequence	Nucleotides
AAATCTGGCAGTGCTCAGGGTCACTGGGATTCAAGGTGAC	3863-3902
CCAGGACGAACGCCACATCCAGAACCATGGAGTTCCTCTT	4700-4739

5   A series of vWF cDNA clones covering the entire GPIb binding
domain were identified and isolated. The cDNA fragments
were subcloned into EcoRI site of pUC-19 (New England
Biolabs, Inc.). One of the subclones, designated pvW1P
(Figure 1), contains a 2.5 Kb insert. This 2.5 Kb insert
10   covers the entire GPIb binding domain extending from 550 bp
upstream of the GPIb binding site to 1100 bp downstream of
the GPIb binding site. (The subclone pvW1P has also been
designated pvWF-1P).

15   Manipulation of DNA Coding for the vWF GPIb Binding Domain

In order to obtain expression of the GPIb binding domain in
Escherichia coli under the regulation of the deo P_1P_2
promoter, the cDNA fragment of vWF, derived from plasmid
20   pvW1P was used for further manipulations as described below.
As indicated previously, the vWF tryptic digest fragment
that binds the GPIb receptor is from amino acid Val 449 to
amino acid Lys 728.

25   A. Subcloning of the 5' end of vWF GPIb binding domain and
    addition of a translation initiation codon ATG.

Plasmid pvW1P has two convenient restriction sites at the 5'
end. Bsu36I which cuts at the DNA sequence corresponding to
30   amino acid Ser (445), and Tth111I which cuts at amino acid
Asp (514). Synthetic fragments of various size were
designed that insert an ATG translation initiation codon at
the 5' end as well as additional amino acids. This was done
first, in ord r to maximize the chances of obtaining high
35   levels of expression. Second, they are a first step towards

reducing the size of the vWF GPIb binding domain peptide down to the minimal size needed, possibly eliminating collagen and heparin binding sites which may ultimately interfere with the function of the product.

A1.   Amino acid Glu 437 at 5' end

Synthetic oligomers with the sequences:

```
5' - TATGGAGGTGGCTGGCCGGCGTTTTGCC - 3'
3' - ACCTCCACCGACCGGCCGCAAAACGGAGT - 5'
     NdeI                        Bsu36I
```

were ligated to plasmid pvWF-1P digested with NdeI and Bsu36I (see Figure 2). The plasmid obtain d was designated pvWF-VA1. Plasmid pvWF-VA1 has be n maintained in E. coli strain Sφ930 and was deposited under ATCC Accession No. 68530.

A2.   Amino acid Phe 443 at 5' end

Synthetic oligomers with the sequences:

```
5' - TATGTTTGCC - 3'
3' - ACAAACGGAGT - 5'
```

were ligated to plasmid pvW1P digested with NdeI and Bsu36I (see Figure 3). The plasmid obtained was designated pvWF-VB1.

B. Subcloning of the 3' end of vWF GPIb binding domain,
   introduction of translation stop codon.

5   B1. Introduction of stop codon in plasmid pvWF-VA1

       A synthetic oligomer with the sequence:

          5'-CCGGGGCTCTTGGGGGTTTCGACCCTGGGGCCCAAGTAAGATATCA-3'
10   3'-CCGAGAACCCCCAAAGCTGGGACCCCGGGTTCATTCTATAGTTCGA-5'

          was ligated to an XmaI and HindIII digested plasmid
          pvWF-VA1 (see Figure 4). The plasmid obtained was
          designated pvWF-VA2. This newly constructed
15   plasmid contains a translation termination codon
          TAA adjacent to amino acid 728 (Lys) and EcoRV
          site.

       B2. Introduction of translation stop codon in plasmid
20   pvWF-VB1

       A synthetic oligomer with the sequence:

          5'- CCGGGGCTCTTGGGGTTTCGACCCTGGGGCCCAAGTAAGATATCA - 3'
25   3' - CCGAGAACCCCCAAAGCTGGGACCCCGGGTTCATTCTATAGTTCGA - 5'

          was ligated to plasmid pvWF-VB1 digested with XmaI and
          HindIII. The plasmid obtained was designated pvWF-VB2
          (see Figure 5).

30
Expression of the vWF GPIb binding domain in Escherichia
coli

In order to obtain expression of the vWF GPIb binding domain
35 various expression plasmids were constructed based on a deo

P₁P₂ constitutive promoter system.

1.   <u>Expression of a vWF GPIb binding domain polypeptide
      including amino acid Glu 437 to amino acid Lys 728
      (based on plasmid pvWF-VA2)</u>

An NdeI-EcoRV fragment was isolated from plasmid pvWF-VA2
and ligated into plasmid pMF-945 (see Figure 11) digested
with NdeI and PvuII (see Figure 6). The plasmid obtained
was designated as pvWF-VA3 and was maintained in
<u>Escherichia coli</u> strain Sφ930.

2. <u>Expression of a vWF GPIb binding domain polypeptide
    including amino acid Phe 443 to amino acid Lys 728 (based
    on plasmid pvWF-VB2)</u>

An NdeI-EcoRV fragment was isolated from plasmid pvWF-VB2
and ligated into plasmid pMF-945 digested with NdeI and
PvuII (see Figure 7).   The plasmid obtained was
designated as pvWF-VB3 and was maintained in <u>Escherichia
coli</u> strain Sφ930.

3. <u>Expression of a vWF GPIb binding domain polypeptide
    including amino acid Leu 504 to amino acid Lys 728 (based
    on expression plasmid pvWF-VA3)</u>

A synthetic oligomer with the sequence:

5' - TA<u>TG</u>TTGCACGATTTCTACTGCAGCAGGCTACTGGACC - 3'
 3' - ACAACGTGCTAAAGATGACGTCGTCCGATGAC<u>CTGGA</u> - 5'
        NdeI   Tth111I

was ligated to plasmid pvWF-VA3 digested with NdeI and
Tth111I. The plasmid obtained was designated as pvWF-VC3
(see Figure 8). Plasmid pvWF-VC3 is maintained in <u>Esche-</u>
<u>richia coli</u> strain Sφ930 and has been deposited with the
ATCC under Accession No. 68241 (also see Figure 13).

4. <u>Expression of a vWF GPIb binding domain polypeptide</u>
<u>including amino acid Leu 513 to amino acid Lys 728 (based</u>
<u>on expression plasmid pvWF-VA3)</u>

A synthetic oligomer with the sequence:

5' - TA<u>TG</u>CTGGACC - 3'
  3'- <u> ACGAC<u>CTGGA</u></u> - 5'
        NdeI   Tth111I

was ligated to plasmid pvWF-VA3 digested with NdeI and
Tth111I. The plasmid obtained was designated pvWF-VD3
(see Figure 9).   Plasmid pvWF-VD3 is maintained in
<u>Escherichia coli</u> strain Sφ930.

<u>Expression of vWF-GPIb binding domain polypeptides</u>

The relative alignment of the expression plasmids is shown
in Figure 10.   Plasmids pvWF-VA3, pvWF-VB3, pvWF-VC3 and
pvWF-VD3 in <u>Escherichia coli</u> strain Sφ930 were used in order
to analyze the levels of expression of the various vWF-GPIb
binding domain peptides. The clones obtained were grown in
LB medium containing Amp (100 µg/ml) at 37°C for 48 hours.

After 48 hours growth bacterial cells were harvested and
centrifuged for 2 minutes at 10,000 RPM.   Pellets were
dissolved in 1/10 volume of 50 mM Tris-HCl pH=8.0. Sample

buffer (containing SDS and β-mercaptoethanol) was added.
Samples were boiled for 10 minutes and loaded on a 10% SDS
polyacrylamide gel. The expression of the vWF GPIb binding
domain polypeptides in clones pvWF-VA3, pvWF-VB3 and pvWF-
5   VD3 was low relative to the bacterial total proteins. The
vWF polypeptides from these clones were detectable by
Western blot analysis using commercially available
polyclonal vWF antibody (Dekopatts a/s, Glostrup, Denmark).
However, clones originated from Escherichia coli strain
10   Sφ930 transformed with plasmid pvWF-VC3 expressed the vWF
GPIb binding domain polypeptide (amino acid Leu 504 to amino
acid Lys 728 plus methionine) at high levels (as a major
band) detectable upon Coomassie staining.

15   Escherichia coli strain Sφ930 harboring plasmid pvWF-VC3 was
deposited with the ATCC under Accession No. 68241.
Subsequently, an inducible plasmid was constructed which
contains the same vWF coding region as pvWF-VC3, express d
under the control of the λ P_L promoter and the deo ribosomal
20   binding site (see Figure 14). This new plasmid, designat d
pvWF-VCL, proved to be a high expressor of VCL, the vWF GPIb
binding domain polypeptide (methionine plus amino acid L u
504 to amino acid Lys 728). This plasmid was deposited in
Escherichia coli strain 4300 with the ATCC under Accession
25   No. 68242. Escherichia coli strain 4300, constructed from
Escherichia coli strain ATCC Accession No. 12435, is a wild-
type, F⁻, biotin dependent strain, harboring the λ cI857
temperature-sensitive repressor. (A third plasmid construct
harboring the same vWF coding region under the control of
30   the λ promoter and the cII ribosomal binding site did not
express any vWF peptide detectable by Coomassie staining.)

The NdeI-HindIII insert of pvWF-VCL can be conveniently
35   subcloned into other expression vectors such as commercially

available pUC19 for production of a series of polypeptides
which include the same amino acid sequence from amino acid
509 (cys) to amino acid 695 (cys) and have the same
biological activity.

Example 2:   Fermentation of Bacteria Expressing vWF GPIb
                   Binding Domain Polypeptides

During scale-up fermentations of clone pvWF-VC3 it was found
that the host tends to lose the plasmid due to instability.
The loss of plasmids caused a reduction in vWF GPIb binding
domain polypeptide expression. It was found necessary to
maintain continuous selective pressure (i.e., continuous
addition of Ampicillin) in order to maintain plasmid copy
number and to maintain the expression levels. Large scale
fermentation was carried out for 12 hours.

Fermentation was carried out in the following growth medium:

N-Z amino AS	20 gr
Yeast extract	10 gr
NaCl	5 gr
K_2HPO_4	2.5 gr
$MgSO_47H_2O$	1.0 gr
Anti foam	0.4 ml

Fructose (50%) was added to the growth medium at final
concentration of 150 ml/liter and Ampicillin (100 mg/ml) was
pumped continuously into the fermentor (total of 8
ml/liter). Fermentation was carried out for 12 hours at
37°C.

Purification of polypeptides

Cells were harvested after 12 hours fermentation and centri-
fuged. The bacterial pellet obtained was resuspended in
buffer [50 mM Tris pH=8.0, 50 mM NaCl, 1 mM EDTA, 1mM DTT
(dithiothreitol), 1 mM PMSF (Phenylmethylsulfonyl fluoride)
and 10% Glycerol]. After additional centrifugation and
sonication the vWF GPIb binding domain polypeptide was found

-34-

in the pellet.

The vWF GPIb binding domain polypeptide was further purified
by solubilization of the pellet in 8M Urea containing 10 mM
5   DTT, 25 mM Tris pH=8 and 1 mM EDTA at room temperature. The
solubilized pellet was fractionated on a DEAE cellulose ion
exchange column chromatography. (Elution buffer as above
except 0.5 mM DTT).

10   The vWF GPIb binding domain polypeptide was eluted at 150 mM
NaCl. After dilution to 50 mM NaCl (in the above buffer)
the partially purified peptide was loaded on a Q-Sepharose
column. Elution from the Q-Sepharose column was carried out
at various NaCl concentrations (step elution). The vWF GPIb
15   binding domain peptide was pooled in four peaks which eluted
at 100 mM, 200 mM, 250 mM and 500 mM NaCl. All four peaks
were dialyzed against 150 mM NaCl and 50 mM Tris pH=8 for 36
hours. During the dialysis the Urea concentration of the
dialysis solution was reduced in a linear gradient from 6M
20   Urea to no Urea.

Exampl 3:   **Biological Activity of vWF GPIb Binding Domain**
                Polypeptides

Platelet Aggregation Assays

vWF preparation:

Human plasma-derived vWF was purified from human outdated
blood bank plasma according to J. Loscalzo and R.I. Handin,
Biochemistry 23: 3880-3886 (1984). The purified plasma-
derived vWF was concentrated by Amicon 100,000 cut-off
filter membrane, to a final concentration of 0.25 mg/ml.

Asialo-vWF preparation:

The purified plasma-derived human vWF was desialyated
according to L. DeMarco and S. Shapiro, J. Clin. Invst. 68:
321-328 (1981) with the following modifications:

1.   The Neuraminidase used was from Vibrio cholera type
      II (Sigma).

2.   The reaction mixture contained 0.2 Units enzyme/ mg
      protein and protease inhibitors according to the
      following concentrations: Benzamidine (20 mM),
      Leupeptin (15 µg/ml) and Aprotinin 20 (U/ ml). The
      asialo-vWF was used for platelet aggregation with-
      out any further purification.

Platelet aggregation - Induced by Asialo-vWF

As stated above, soluble vWF does not bind to platelets via
the GPIb receptor. Asialo-vWF, obtained by neuraminidase
treatment to remove sialic acid residues, readily binds to
platelets via GPIb. Presumably, the d sialation lowers the

net negative charge on the vWF, allowing it to bind to th negatively charged GPIb receptor. Asialo-vWF binding to platelets causes activation, release of ADP, and GP IIb/ IIIa mediated aggregation. Platelet aggregation induced by asialo-vWF was carried out with 200 µl of PRP (Platelet-rich plasma) (Fujimura Y., et al., J. Biol. Chem. _261_: 381-385 (1986)) and 39 µg/ml of asialo-vWF (final concentration) in a Lumi aggregometer. The results of inhibition of platelet aggregation with VC, the vWF GPIb binding domain polypeptide, are summarized in Table I.

VC (also referred to as VCL or VC3) is a vWF GPIb binding domain polypeptide which includes methionine plus amino acids 504-728 (see Figure 12).

vWF-Ristocetin induced platelet aggregation

Ristocetin-induced platelet aggregation in the presence f purified human intact vWF was carried out with washed human platelets according to Fujimura Y. et al., J. Biol. Chem. _261_: 381-385 (1986).

The results of inhibition of platelet aggregation induced by ristocetin in the presence of intact vWF are summarized in Table II. Additional results using these assays are described in Example 5.

TABLE I

Inhibition of Asialo-vWF Induced Platelet Aggregation
(In PRP) by VC, a vWF GPIb Binding Domain Polypeptide

Q-Sepharose Fraction	VC concentration μM	% Inhibition of Platelet Aggregation	
200 mM NaCl	6	76	64
250 mM NaCl	6	82	73
500 mM NaCl	6	89	79

TABLE II

Inhibition of Ristocetin Induced Platelet Aggregation by VC, a vWF GPIb Binding Domain Polypeptide

Q-Sepharose Fraction	VC concentration in μM	% Inhibition of Platelet Aggregation
200 mM NaCl	10	76
	6	69
	3	38
	1	22
250 mM NaCl	10	86
	6	67
	3	44
	1	34
500 mM NaCl	10	100
	6	79
	3	68
	1	54
	0.25	38
Dialysis Buffer (control)	0	0

Exampl 4:

An Improved Method of Obtaining Pure, Oxidized, Folded and Biologically Active vWF GPIb Binding Domain Polypeptide

In Example 2, fermentation of cells harboring plasmid pvWF-VC3 was described. Subsequently, a preferred plasmid, pvWF-VCL was constructed as described in Example 1 and maintained in E.coli strain A4300. This host/plasmid system was fermented essentially as known in the art for vectors containing a gene expressed under control of the λP_L promoter, see, for example coassigned EPO Patent Publication No. 173,280, published March 5, 1986, Example 5, pages 73-74 (without added biotin, thiamine, trace elements, and ampicillin). In this improved method of purification of vWF GPIb binding domain polypeptide, a cell pellet of the above fermentation of A4300/pvWF-VCL was used.

In this improved method a purer and more active polypeptide is produced than by the method disclosed in Example 2. The general scheme of the downstream process consists of steps A through H as follows:

A. **Cell disruption and suspension of pellet**: A pellet containing the vWF GPIb binding domain polypeptide is obtained as described in Example 2, by sonication and centrifugation of a cell suspension in 50mM Tris-HCl pH=8, 50mM NaCl, 1mM EDTA, 1mM DTT, 1mM PMSF, and 10% Glycerol.

The pellet containing the inclusion bodies is dissolved at about 10% w/v in a solution such that the final concentrations after dissolution are 8M urea, 20mM DTT, 20mM HEPES pH 8, and 100mM NaCl. The resulting solution may be further purified by ion exchang chromatography as

described below. Alternatively, the inclusion bodies may be solubilized in a buffer containing 6M guanidine hydrochloride followed by buffer exchange to urea. The inclusion bodies may also be dissolved at different concentrations of urea, guanidine hydrochloride or any other denaturant or in the absence of denaturants, for example, at extremes of pH.

B. Cation exchange chromatography: This step eliminates most of the contaminants and produces the vWF GPIb binding domain polypeptide at greater than 90% purity. Any cation exchange (e.g. carboxymethyl) method may be used in this step, but CM-Sepharose fast flow (Pharmacia) chromatography is preferred. The functional group may be carboxymethyl, a phospho group or sulphonic groups such as sulphopropyl. The matrix may be based on inorganic compounds, synthetic resins, polysaccharides, or organic polymers; possible matrices are agarose, cellulose, trisacryl, dextran, glass beads, oxirane acrylic beads, acrylamide, agarose/polyacrylamide copolymer (Ultrogel) or hydrophilic vinyl polymer (Fractogel). In a specific embodiment, the polypeptide is loaded onto a CM-Sepharose FF column equilibrated with 8M urea, 1mM DTT, 20mM HEPES pH 8, 100mM NaCl. Pure polypeptide elutes in 8M urea, 1mM DTT, 20mM HEPES pH 8 and 200mM NaCl. Up to about 30 OD_{280} units of solubilized inclusion bodies may be loaded per ml CM-Sepharose FF. At this ratio the eluted polypeptide typically has a concentration of 4-5 OD_{280}/ml.

C. Oxidation/Refolding: The polypeptide solution eluted from the cation exchange step above is treated with 6M guanidine hydrochloride (GuCl) to disrupt any aggregates. The polypeptide is then dilut d to 0.05 OD_{280}/ml in 2M GuCl, pH 5-11, preferably 20mM HEPES pH 8, 0.1mM GSSG

(glutathione, oxidized form). This mixtur is allowed to
stand ov rnight at room temperature. The products ar
analyzed by gel filtration on fast protein liquid
chromatography (FPLC) such as Superose 12 before
5   proceeding. Analysis shows that this protein
concentration reproducibly yields about 30% correctly
oxidized monomers, and 70% S-linked dimers and multimers,
as well as reduced and incorrectly oxidized monomers. A
higher protein concentration gives a higher absolute
10 yield of correctly oxidized monomers but a low r
percentage yield due to increased formation of S-linked
dimers and multimers. For example, a protein
concentration of 0.1 OD_{280}/ml yields only 20% correctly
oxidized monomers. Reducing the concentration to 0.025
15 OD_{280}/ml yields 35-40% correctly oxidized monomers but a
lower absolute yield per liter oxidation. Oxidations may
also be performed in urea instead of in GuCl, or in any
other denaturant or in the absence of denaturants under
appropriate buffer conditions in which, for example, pH,
20 ionic strength, and hydrophobicity are varied. The
preferred concentration of urea is in the range 0.5M to
10M, preferably 4M, and the preferred oxidant is GSSG in
the range 0.01mM to 5mM preferably 0.1mM. Other oxidants
such as $CuCl_2$ may be used or alternatively no oxidant may
25 be added, thereby utilizing air oxidation only. For
scale-up, 4M urea is the presently preferred solution for
the oxidation step.

D. Concentration: The oxidation products are concentrated,
30 preferably to about OD_{280}=1 by a tangential flow ultra-
filtration system with a 30K cutoff membrane, such as a
"MINITAN" or "PELLICON" system of Millipore. The
filtrat is quit clear as the material is relatively
clean and most of the contaminants ar larg enough not
35 to pass through the 30K membran . It is thus possibl to

reuse the filtrate for performing oxidations. This
results in considerable savings since large volumes of 2M
GuCl are quite expensive. No difference in the oxidation
products of oxidations performed in reused versus freshly
5   prepared 2M GuCl was detectable by FPLC analysis.

E. Dialysis: It is necessary to reduce the GuCl or urea
concentration to less than 10mM. This is achieved by
10   dialysis against 20mM HEPES pH8, 100mM NaCl. The
dialysis was performed in dialysis tubing with 2-3
changes of buffer, but may be alternatively performed by
diafiltration against the same buffer in a tangential
flow ultrafiltration system with a 10K MW cutoff
15   membrane.

During dialysis, as the concentration of GuCl (or urea or
other denaturant) decreases, a white precipitate forms.
20   This precipitate contains about 80% of the protein
yielded by step D comprising S-S linked dimers, reduced
and incorrectly oxidized monomer and some contaminants
which coeluted from the cation exchange step. The
supernatant is nearly 100% correctly oxidized and
25   refolded monomer at a concentration of 0.2 OD_{280}/ml,
which is about 20% of the protein yield of step D. This
selective precipitation of contaminants and undesirable
forms of the protein as a result of dialysis was
surprising and not predictable. The yield of correctly
30   oxidized monomer can be greatly increased by recovery
from the precipitate. This is done as follows: the
solution is clarified by centrifugation. The supernatant
is saved, and the pellet is tr ated with DTT to reduce
S-S bonds and reoxidized as described above. The pellet
35   is dissolved in a minimal volume of 6M GuCl, 20ml HEPES

pH 8, 150mM NaCl, 20mM DTT. The solution was passed through Sephadex G25 in a buff r similar to the dissolution buffer but containing only 1mM DTT (instead of 20mM). The eluate is then diluted to OD_{280}=0.05 and treated as in steps C, D and E above. This procedure may be repeated more than once as long as additional purified monomer is obtained. All of the supernatants are then combined.

F. <u>Cation exchange</u>: The combined supernatant of the dialysate of step E is concentrated by binding to CM Sepharose in 20mM HEPES pH8, 100mM NaCl. Elution is with 20mM HEPES pH8, 400mM NaCl. The eluate is exclusively monomeric despite the high salt concentration. Concentrations of up to 3 mg/ml have been achieved by this method and that is not the upper limit. This st p can alternatively be performed with Heparin-Sepharose which also binds the purified monomer in 10mM Tris pH 7.4, 150mM NaCl. Elution from Heparin-Sepharose is performed using 10mM Tris-HCl pH 7.4, 500mM NaCl.

G. <u>Dialysis</u>: The product of the previous step is dialyzed against 20mM HEPES pH8, 150mM NaCl.

H. <u>Storage</u>: At this stage the purified vWF GPIb binding domain polypeptide may be lyophilized. Upon reconstitution in a volume of water equal to the volume before lyophilization, the resultant solution contains exclusively monomeric protein showing no traces of dimers or other multimers on FPLC.

In a specific embodiment of this method the following procedure was performed:

a)   10 gm inclusion bodies (comprising 0.43 g net dry weight) were dissolved in a final volume of 100ml 8M urea, 20mM DTT, 20mM HEPES pH 8, 100 mM NaCl.

5   b)   The protein was loaded onto a CM Sepharose column equilibrated with 8M urea, 1mM DTT, 20mM HEPES pH 8, 100mM NaCl.   The protein eluted at 200mM NaCL in 8M urea, 20mM HEPES pH 8, 1mM DTT, and was saved.

10   c)   The saved eluate of the previous step was treated with 6M GuCl to eliminate any aggregates, and was then diluted to 0.05 OD_{280}/ml in 2M GuCl, 20mM HEPES pH 8, 0.1mM GSSG. Oxidation was performed overnight at room temperature. (Note that the oxidation step can be performed in the
15   presence of urea instead of GuCl.)

d)   The oxidation products were concentrated to OD_{280}=1 by ultrafiltration on a "MINITAN" unit containing a 30K membrane.
20

e)   The concentrate of the previous step was dialyzed with three buffer changes against 20mM HEPES pH 8, 100mM NaCl. During dialysis, as the GuCl concentration decreased, a white precipitate formed which was removed by centrifugation
25   and reprocessed once as described above. The supernatants were combined.

f)   The combined supernatants were concentrated by binding
30   to CM Sepharose in 20mM HEPES pH 8, 100mM NaCl.   The polypeptide was eluted in 20mM HEPES, pH 8, 400mM NaCl and stored at 4°C.

35   g)   The saved eluate from th previous st p was dialyzed

against 20mM HEPES pH 8, 150mM NaCl at 4°C.

h)   After dialysis, the purified vWF GPIb binding domain
polypeptide, designated VCL, was lyophilized.

5

Analysis of VCL

1.   Amino acid sequence analysis of VCL purified as
described above revealed that the N-terminal sequence is
Met-Leu-His-Asp-Phe which is the expected sequence according
10   to Figure 12 with the addition of an N-terminal methionine
residue.

2.   Examination of VCL on polyacrylamide gels revealed that
VCL electrophoreses at lower apparent molecular weight und r
15   non-reducing conditions than under reducing conditions
(beta-mercaptoethanol). This shift from compact to less
compact configuration is consistent with the reduction of a
disulfide bond. Such an intramolecular bond is formed
between the cysteines at positions 509 and 695. (The shift
20   in molecular weight is not large enough to be consistent
with the reduction of an intermolecular bond.)

Example 5:

Biological Activity of VCL, a vWF GPIb binding domain polypeptide

5   The vwF GPIb binding domain polypeptide produced as described in Example 4 was designated VCL and was assayed for biological activity as described below.

10   1.   **Ristocetin induced platelet aggregation (RIPA)**

       RIPA assay was performed as described in Example 3 in a reaction mix containing 2×10^8 platelets/ml, $1\mu g$/ml plasma vWF, and 1mg/ml ristocetin. A series of concentrations of
15   VCL was tested and the IC_{50} of VCL in 3 assays was determined to be $0.2-0.3\mu M$. 100% inhibition was achieved with about $1\mu M$ VCL.

       2.   **Asialo vWF induced platelet aggregation**
20
       Asialo vWF induced platelet aggregation assay was perform d as described in Example 3 with $200\mu l$ platelet-rich plasma (PRP) and $10\mu g$/ml asialo-vWF in a Lumi aggregometer. A series of concentrations of VCL was tested and the IC_{50} of
25   VCL in this assay was determined to be $0.15\mu M$, and complete inhibition by 0.5 μM.

       3.   **Effect of VCL on preformed aggregates**

30   The effect of VCL on preformed aggregates made by RIPA was tested. Aggregates were formed as in paragraph (1) above in the absence of VCL. Addition of VCL to a concentration of $0.5\mu M$ disrupted the aggregat s instantaneously.

35

4.   Inhibition of thrombin induced platelet aggregation

Thrombin induced platelet aggregation assay was performed
using 0.025 unit/ml thrombin and stractan prepared
platelets. A series of concentrations of VCL was tested and
the IC_{50} of VCL in this assay was determined to be 0.3μM.
This is a surprising effect, since in a parallel experiment,
VCL was not effective in inhibiting direct binding of [^{125}I]
labelled thrombin to platelets.

5.   Effect on platelet deposition under conditions of flow

In a model system consisting of an everted denuded human
umbilical artery in a flow cell, platelet deposition may be
determined.   Whole human blood flows over the artery
fragment.   After 10-15 minutes, the flow is stopped and
platelet deposition is determined microscopically. The IC_{50}
of VCL in this system was determined to be about 1μM.

All the above results are summarized in Table III.

The inhibitory activity of VCL on ristocetin-induced or
asialo vWF-induced platelet aggregation, ristocetin-induced
vWF binding, and platelet adhesion was lost upon reduction
of the disulfide bond between the cysteines at positions 509
and 695. In some experiments, the reduced VCL precipitated
out of solution.

TABLE III

Biological Activity of VCL,
a vWF GPIb Binding Domain Polypeptide.

Example 5 (Paragraph No.)	Assay	μM VCL
1	Ristocetin induced platelet aggregation	$IC_{50}=0.2-0.3$
2	Asialo vWF induced platelet aggregation	$IC_{50}=0.15$
3	Thrombin induced platelet aggregation	$IC_{50}=0.3$
4	Dissolution of preformed aggregates	0.5
5	Platelet deposition under conditions of flow	$IC_{50}=1$

Exampl 6:

Construction of Plasmid pvWF-VEL

It was decided to construct a plasmid which expresses a
slightly longer portion of the vWF GPIb binding domain than
pvWF-VCL. The construction is shown in Figures 15-18 and
described in the brief descriptions of the figures.

A.   Construction of pvWF-VE2

Plasmid pvWF-VA2 (constructed as shown in Figure 4) was
digested with NdeI and PstI and the large fragment isolated.
Four synthetic oligomers shown in Figure 16 were prepared.
Nos. 2 and 3 were treated with T4 polynucleotide kinase to
add 5' phosphate. The above mentioned large fragment of
pvWF-VA2 was ligated as shown in Figure 15 with the four
oligomers (two kinased, and two non-kinased). The resulting
plasmid shown in Figure 15 was designated pvWF-VE2.

B.   Construction of plasmid pvWF-VE3

Plasmid pvWF-VE2 was digested with NdeI and HindIII and the
770 bp fragment containing the vWF GPIb binding domain was
isolated. Plasmid pMLK-7891 was also digested with NdeI and
HindIII and the large fragment was isolated. The resulting
plasmid, shown in Figure 17, was designated pvWF-VE3.

C. Construction of plasmid pvWF-VEL

Plasmid pvWF-VE3 was digested with XmnI, dephosphorylated
with bacterial alkaline phosphatase (BAP) and then digested
with NdeI and HindIII. Plasmid pMLK-100 was digested with
NdeI and HindIII and dephosphorylated with BAP. The two
digests were th n ligated to yield plasmid pvWF-VEL as shown
in Figure 18. This plasmid expresses the DNA sequence

corresponding to amino acids 469-728 of mature vWF under the
control of the λP$_L$ promoter and the cII ribosomal binding
site. The protein probably also includes an additional N-
terminal methionine residue. A conservative base change was
introduced into ala-473 changing GCC to GCA which also
encodes alanine. This introduced an SphI site into the gene
by changing GCCTGC to GCATGC.

Expression of pvWF-VEL in E. coli 4300(F⁻) yields a 29 kD
protein which reacts strongly with a monoclonal anti-vWF
antibody and will be referred to herein as VEL.

Exampl 7:

Pharmaceutical Uses of vWF GPIb Binding Domain Polypeptide

5   Examples 1 and 4 describe the production and purification of
a novel vWF GPIb binding domain polypeptide designated VCL.
Some of the uses envisaged for VCL or for other vWF GPIb
binding   domain   polypeptides   are   described   below.
Pharmaceutical compositions containing VCL or such other
10   polypeptides   may   be   formulated   with   a   suitable
pharmaceutically acceptable carrier using methods and
carriers well known in the art.

1.   The VCL composition described above may be used for
15   prevention of platelet adhesion to damaged vascular surfac s
(see Example 5, sub-section 5).

2.   The VCL composition described above may be used for
disruption of platelet-rich aggregates (see Example 5,
20   subsection 3).

3.   The VCL composition described above may be used for
prevention of re-occlusion following angioplasty or
thrombolysis (see Bellinger et al., PNAS, USA, 84: 8100-8104
25   (1987), Prevention of occlusive coronary artery thrombosis
by a murine monoclonal antibody to porcine von Willebrand
Factor).

4.   The VCL composition described above may be used for
30   prevention of platelet activation and thrombus formation due
to high shear forces such as in stenosed or partially
obstructed arteries or at arterial bifurcations (see
Peterson et al., Blood 2: 625-628 (1987), Shear-induc d
platelet aggregation requires von Willebrand Factor and
35   platelet membrane glycoproteins Ib and IIb-IIIa).

5.   The VCL composition described above may be used for
prevention of thrombosis and re-occlusion after angioplasty
or thrombolysis due to thrombin activation of platelets (see
Fuster et al., J. Am. Coll. Cardiol. 12: 78A-84A (1988),
Antithrombotic therapy after myocardial reperfusion in acute
myocardial infarction).

6.   The VCL composition described above may be used for
prevention of platelet adhesion to and aggregation on
prosthetic materials (see Badimon et al., J. of Biomaterials
Applications, 5: 27-48 (1990), Platelet interaction to
prosthetic materials - role of von Willebrand Factor in
Platelet Interaction to PTFE).

7.   The VCL composition described above may be used for
prevention of intramyocardial platelet aggregation in
patients with unstable angina (see Davies et al.,
Circulation 73: 418-427 (1986), Intramyocardial platelet
aggregation in patients with unstable angina suffering
sudden ischemic cardiac death).

8.   The VCL composition described above may be used for
prevention of vasospasm and vasoconstriction following
arterial injury caused by angioplasty, thrombolysis or other
causes (see Lam et al., Circulation 75: 243-248 (1987), Is
vasospasm related to platelet deposition?) .

9.   The VCL composition described above may be used for
prevention of restenosis following angioplasty or
thrombolysis (see McBride et al., N. Eng. J. of Med. 318:
1734-1737 (1988), Restenosis after successful coronary
angioplasty).

10.   The VCL composition described above may be used for
pr vention of vaso-occlusive crises in sickle-cell anemia

(see Wick et al., J. Clin. Invest. 80: 905-910 (1987),
Unusually large von Willebrand multimers increase adhesion
of sickle erythrocytes to human endothelial cells under
controlled flow).

5

11.   The VCL composition described above may be used for
prevention of thrombosis associated with inflammatory
response (see Esmon, Science 235: 1348-1352 (1987), The
regulation of natural anticoagulant pathways).

10

12.   The VCL composition described above may be used for
prevention of arteriosclerosis (see Fuster et al.,
Circulation Res. 51: 587-593 (1982), Arteriosclerosis in
normal and von Willebrand pigs).

15

13.   The VCL composition described above may be used as
an antimetastatic agent (see Kitagawa et al., Cancer Res.
49: 537-541 (1989), Involvement of platelet membrane
glycoprotein Ib and IIb/IIIa complex in thrombin-dependent
20   and -independent platelet aggregations induced by tumor
cells).

Example 8

1.   In Vitro Studies

5   For these studies VCL, or vehicle control, was made up fresh
in sterile water (2.2 mg/ml stock).

A.   Platelet Aggregation (PRP)

10   This is a standardized von Willebrand Factor (vWF)-dependent
aggregation assay using human or rat platelet rich plasma
(PRP).   The   addition   of   various   concentrations   of
unfractionated Bothrops jararaca venom (BJV), which includes
botrocetin and an additional thrombin-like component, or
15   ristocetin results in an aggregatory response in the absence
of any additional agent. Using ristocetin (1.5 mg/ml) as
the agonist 43 μg/ml VCL abolished the aggregation of human
PRP.   Ristocetin up to 5.0 mg/ml did not cause measurable
aggregation of rat PRP. Using this assay system with BJV as
20   the agonist VCL at 83 μg/ml slightly inhibited the response
of human PRP (Figure 19) but not rat PRP (Figure 20).

It is concluded that ristocetin is not a suitable agonist
for inducing vWF dependent aggregation in the rat. Further,
25   it is not possible to monitor the effects of VCL ex vivo
using BJV-induced aggregation of rat PRP. However, VCL does
inhibit vWF-dependent aggregation in human PRP in vitro.

B. Platelet Thrombin Receptor Assay

30

This assay measures the inhibition of thrombin-induced
platelet pro-coagulant expression and is briefly described
below. Human washed platelets are incubated in a buffer
which contains $CaCl_2$, factor Xa, prothrombin, and human
35   alpha-thrombin for 60 min at 28°C. At the end of this

period an aliquot is transferred into a buffer containing
S2238 and EDTA (to prevent any further thrombin generation).
The S2238 reaction is terminated after 15 minutes at room
temperature with acetic acid and the absorbance at 405 nm

5   read.   The amount of S2238 cleavage directly due to the
added human alpha-thrombin is. estimated by including a
control which contains no prothrombin and this value is
subtracted from all results. VCL was tested in this assay
at a final concentration of 0.1 mg/ml.

10

This assay is sensitive to both thrombin inhibitors and
thrombin receptor antagonists. In the presence of VCL the
thrombin generation was 114% of control (n=2).

15   We therefore conclude that VCL is not a thrombin receptor
antagonist in this system.

2. **In Vivo Studies**

20   Arterial Thrombosis Model (Rat)

This method is essentially a modification of the model of
Shand et al., Thromb. Res. 45 505-515 (1987). The method
we use routinely is outlined below.

25

Rats are labelled with ^{111}In platelets and ^{125}I fibrinogen.
The dorsal aorta is clamped, using modified Spencer-Wells
forceps, for 1 minute. After a 45 minute reperfusion period
the damaged vessel is removed, washed in. citrate and

30   counted. Results are expressed as mg blood equivalents.
Differences in radiolabel accumulation between placebo and
drug-treated animals are calculated and expressed as a
percentag inhibition.

35   For the purpose of the evaluation of VCL the route of

administration was by bolus intravenous injection. VCL was used at doses of 2mg/kg (n=5) and 4mg/kg (n=3). It was administered 1 minute prior to clamping. The vessel was then reperfused for twenty minutes. The antithrombotic

5   effect was assessed at the 20 minute end point of the reperfusion. The shortening of the reperfusion time (as compared to routine) was designed to save compound. Appropriate vehicle controls (n=5 for both doses) were assessed.

10

It can be seen that under these conditions VCL inhibits thrombus formation in this model (Table IV). The inhibition is seen for the platelet (^{III}In) components of the thrombus at the 4mg/kg dose. The other changes do not reach

15   statistical significance, thus VCL shows antithrombotic efficacy in this rat arterial model.

In conclusion, VCL exhibits an antithrombotic effect in this rat model of arterial thrombosis which may be dose

20   dependent.

3.   **Discussion**

From the present data it appears that the VCL interacts with

25   the human platelet vWF receptor and hence inhibits platel t aggregation in human PRP. There is however a marked difference between species (rat vs. human) when comparing inhibition of platelet aggregation. The species specificity of this effect and the causal mechanism were not

30   investigated further. At a practical level this meant we were unable to analyze ex vivo samples in order to correlate the effects of VCL on aggregation and arterial thrombosis. Hence the analysis and interpretation of the in vivo efficacy of VCL as an antithrombotic in the rat arterial

35   thrombosis model is complicated by this factor.

Despite GP1b possessing binding sites for both vWF and
thrombin it would appear that any effects of VCL on thrombin
binding to GP1b do not translate into antagonism of
thrombin-induced pro-coagulant expression.

5

Overall VCL shows an antithrombotic effect in the rat
arterial thrombosis model. This inhibition may be due to
its interference with the binding of vWF to its receptor.

TABLE IV

The Effect of VCL on Arterial Thrombus Formation in the Rat Dorsal Aorta

% INHIBITION					
DOSE (mg/kg)	PLATELETS	P	FIBRINOGEN	P	N
4	61.3 ± 8.0	.01	34.7 ± 8.7	NS	3
2	25.54 ± 20.98	NS	22.78 ± 13.48	NS	5

The results are expressed as mean percentage inhibition ± standard error. The number of experiments in the treated groups are denoted in the table and in all cases were compared to a group of 5 control animals. Statistical analysis was performed on the raw data prior to transformation to percentage inhibition. NS = not statistically significant.

What is claimed is:

1.   A non-glycosylated, biologically active polypeptide
        having the amino acid sequence:

5

        X-A-[Cys Ser Arg Leu Leu Asp Leu Val Phe Leu Leu Asp Gly
        Ser Ser Arg Leu Ser Glu Ala Glu Phe Glu Val Leu Lys Ala
        Phe Val Val Asp Met Met Glu Arg Leu Arg Ile Ser Gln Lys
        Trp Val Arg Val Ala Val Val Glu Tyr His Asp Gly Ser His
10   Ala Tyr Ile Gly Leu Lys Asp Arg Lys Arg Pro Ser Glu L u
        Arg Arg Ile Ala Ser Gln Val Lys Tyr Ala Gly Ser Gln Val
        Ala Ser Thr Ser Glu Val Leu Lys Tyr Thr Leu Phe Gln Ile
        Phe Ser Lys Ile Asp Arg Pro Glu Ala Ser Arg Ile Ala Leu
        Leu Leu Met Ala Ser Gln Glu Pro Gln Arg Met Ser Arg Asn
15   Phe Val Arg Tyr Val Gln Gly Leu Lys Lys Lys Lys Val Ile
        Val Ile Pro Val Gly Ile Gly Pro His Ala Asn Leu Lys Gln
        Ile Arg Leu Ile Glu Lys Gln Ala Pro Glu Asn Lys Ala Ph
        Val Leu Ser Ser Val Asp Glu Leu Glu Gln Gln Arg Asp Glu
        Ile Val Ser Tyr Leu Cys]-B-COOH

20

        wherein X is NH₂-methionine- or NH₂-;

        A is a sequence of at least 1, but less than 35
        amino acids, which sequence is present in naturally
25   occurring human vWF, the carboxy terminal amino
        acid of which is the tyrosine #508 shown in Figure
        12;

        B is a sequence of at least 1, but less than 211
        amino acids, which sequence is present in naturally
30   occurring human vWF, the amino terminal amino acid
        of which is the aspartic acid #696 shown in Figure
        12; and

35   the two cysteines included within the bracketed

sequence are joined by a disulfide bond.

2.   A polypeptide of claim 1 having the amino acid
        sequence:

X-[Leu His Asp Phe Tyr Cys Ser Arg Leu Leu Asp Leu Val
Phe Leu Leu Asp Gly Ser Ser Arg Leu Ser Glu Ala Glu Phe
Glu Val Leu Lys Ala Phe Val Val Asp Met Met Glu Arg Leu
Arg Ile Ser Gln Lys Trp Val Arg Val Ala Val Val Glu Tyr
His Asp Gly Ser His Ala Tyr Ile Gly Leu Lys Asp Arg Lys
Arg Pro Ser Glu Leu Arg Arg Ile Ala Ser Gln Val Lys Tyr
Ala Gly Ser Gln Val Ala Ser Thr Ser Glu Val Leu Lys Tyr
Thr Leu Phe Gln Ile Phe Ser Lys Ile Asp Arg Pro Glu Ala
Ser Arg Ile Ala Leu Leu Leu Met Ala Ser Gln Glu Pro Gln
Arg Met Ser Arg Asn Phe Val Arg Tyr Val Gln Gly Leu Lys
Lys Lys Lys Val Ile Val Ile Pro Val Gly Ile Gly Pro His
Ala Asn Leu Lys Gln Ile Arg Leu Ile Glu Lys Gln Ala Pro
Glu Asn Lys Ala Phe Val Leu Ser Ser Val Asp Glu Leu Glu
Gln Gln Arg Asp Glu Ile Val Ser Tyr Leu Cys Asp Leu Ala
Pro Glu Ala Pro Pro Pro Thr Leu Pro Pro Asp Met Ala Gln
Val Thr Val Gly Pro Gly Leu Leu Gly Val Ser Thr Leu Gly
Pro Lys]-COOH

wherein X is NH_2-methionine- or NH_2-.

3.   A pharmaceutical composition comprising an amount
        of a polypeptide of claim 1 or 2 effective to in-
        hibit platelet aggregation and a pharmaceutically
        acceptable carrier.

4.   A method of inhibiting platelet aggregation which
        comprises contacting platelets with an amount of a
        polyp ptide of claim 1 or 2 effective to inhibit
        platelet aggregation so as to inhibit platelet
        aggregation.

5.   An expression plasmid encoding a polypeptide of
        claim 1.

6.   An expression plasmid encoding a polypeptide of
        claim 2 designated pvWF-VC3 and deposited under
        ATCC Accession No. 68241.

7.   An expression plasmid encoding a polypeptide of
        claim 2 designated pvWF-VCL deposited under ATCC
        Accession No. 68242.

8.   A bacterial cell which comprises the expression
        plasmid of claim 5, 6, or 7.

9.   An Escherichia coli cell of claim 8.

10.   A method of producing a polypeptide of claim 1
        which comprises transforming a bacterial cell with
        an expression plasmid encoding the polypeptide,
        culturing the resulting bacterial cell so that the
        cell produces the polypeptide encoded by the plas-
        mid, and recovering the polypeptide so produced.

11.   A method of producing a polypeptide of claim 2
        which comprises transforming a bacterial cell with
        an expression plasmid encoding the polypeptide,
        culturing the resulting bacterial cell so that the
        cell produces the polypeptide encoded by the plas-
        mid, and recovering the polypeptide so produced.

12.   A method of treating a subject with a cerebrovascu-
        lar disorder which comprises administering to the
        subject an amount of a polyp ptide of claim 1 or 2
        effective to inhibit platelet aggr gation.

13.   A method of treating a subject with a cardiovascu-
        lar disorder which comprises administering to the
        subject an amount of a polypeptide of claim 1 or 2
        effective to inhibit platelet aggregation.

5

14.   A method of treating a subject in accordance with
        claim 13, wherein the cardiovascular disorder
        comprises acute myocardial infarction.

10   15.   A method of treating a subject in accordance with
        claim 13, wherein the cardiovascular disorder
        comprises angina.

16.   A method of inhibiting platelet aggregation in a
15   subject prior to, during, or after the subject has
        undergone angioplasty, thrombolytic treatment, or
        coronary bypass surgery which comprises administer-
        ing to the subject an amount of a polypeptide of
        claim 1 or 2 effective to inhibit platelet
20   aggregation.

17.   A method of maintaining blood vessel patency in a
        subject prior to, during, or after the subject has
        undergone coronary bypass surgery, which comprises
25   administering to the subject an amount of a poly-
        peptide of claim 1 or 2 effective to inhibit plate-
        let aggregation.

18.   A method of treating a subject having cancer which
30   comprises administering to the subject an amount of
        a polypeptide of claim 1 or 2 effective to retard
        tumor metastasis.

19.   A method of inhibiting thrombosis in a subject
35   which comprises administering to the subject an

amount of a polypeptide of claim 1 or 2 effective
to inhibit the thrombosis.

20. A polypeptide in accordance with claim 1 or 2 bound
to a solid matrix.

21. A method of treating a subject suffering from
platelet adhesion to damaged vascular surfaces
which comprises administering to the subject an
amount of the polypeptide of claim 1 or 2 effective
to inhibit platelet adhesion to damaged vascular
surfaces.

22. A method of preventing platelet adhesion to a
prosthetic material or device in a subject which
comprises administering to the subject an amount of
the polypeptide of claim 1 or 2 effective to
prevent platelet adhesion to the material or
device.

23. A method of inhibiting re-occlusion in a subject
following angioplasty or thrombolysis which com-
prises administering to the subject an amount of
the polypeptide of claim 1 or 2 effective to inhib-
it re-occlusion.

24. A method of preventing vaso-occlusive crises in a
subject suffering from sickle cell anemia which
comprises administering to the subject an amount of
the polypeptide of claim 1 or 2 effective to pre-
vent vaso-occlusive crises.

25. A method of preventing arteriosclerosis in a sub-
ject which comprises administering to the subject
an amount of the polypeptide of claim 1 or 2 effec-

tive to pr vent arteriosclerosis.

26.   A method of thrombolytic treatment of thrombi-
       containing, platelet-rich aggregates in a subject
       which comprises administering to the subject an
       amount of the polypeptide of claim 1 or 2 effective
       to treat thrombi-containing, platelet-rich aggre-
       gates.

27.   A method of preventing platelet activation and
       thrombus formation due to high shear forces in a
       subject suffering from stenosed or partially ob-
       structed arteries which comprises administering to
       the subject an amount of the polypeptide of claim 1
       or 2 effective to prevent platelet activation and
       thrombus formation.

28.   A method of preventing thrombin-induced platelet
       activation in a subject which comprises administer-
       ing to the subject an amount of the polypeptide of
       claim 1 or 2 effective to prevent thrombin-induced
       platelet activation.

29.   A method of preventing stenosis as a result of
       smooth muscle proliferation following vascular
       injury in a subject which comprises administering
       to the subject an amount of the polypeptide of
       claim 1 or 2 effective to prevent stenosis.

30.   A method of claim 19, wherein the thrombosis is
       associated with an inflammatory response.

31.   A method for recovering a purified, biologically
       activ polyp ptide of claim 1 which comprises:

(a) producing in a bacterial cell a first
     polypeptide having the amino acid
     sequence of the polypeptide but lacking
     the disulfide bond;

5

(b) disrupting the bacterial cell so as to
     produce a lysate containing the first
     polypeptide;

10

(c) treating the lysate so as to obtain
     inclusion bodies containing the first
     polypeptide;

(d) contacting the inclusion bodies from step

15   (c) so as to obtain the first polypeptide
     in soluble form;

(e) treating the resulting first polypeptide
     so as to form the biologically activ

20   polypeptide;

(f) recovering the biologically activ
     polypeptide so formed; and

25   (g) purifying the biologically activ
     polypeptide so recovered.

32. A method of claim 31, wherein the treatment step
     (e) comprises contacting the polypeptide with a

30   thiol-containing compound and disulfide.

33. A method of claim 32, wherein the thiol-containing
     compound is glutathione, thioredoxin, ß-
     mercaptoethanol or cysteine.

35

34.   A method of claim 31, wherein the contacting of step (d) is effected in th presence of a denaturant.

5   35.   A method of claim 34, wherein the denaturant is guanidine hydrochloride or urea.

36.   A method of claim 34, wherein the recovery of the polypeptide of step (f) comprises removing the

10   denaturant by dialysis.

37.   A method of claim 31, wherein in step (g) the polypeptide is purified by cation exchange chromatography.

15

38.   A method of claim 37, wherein the first polypeptide is purified by cation exchange chromatography after step (d).

20
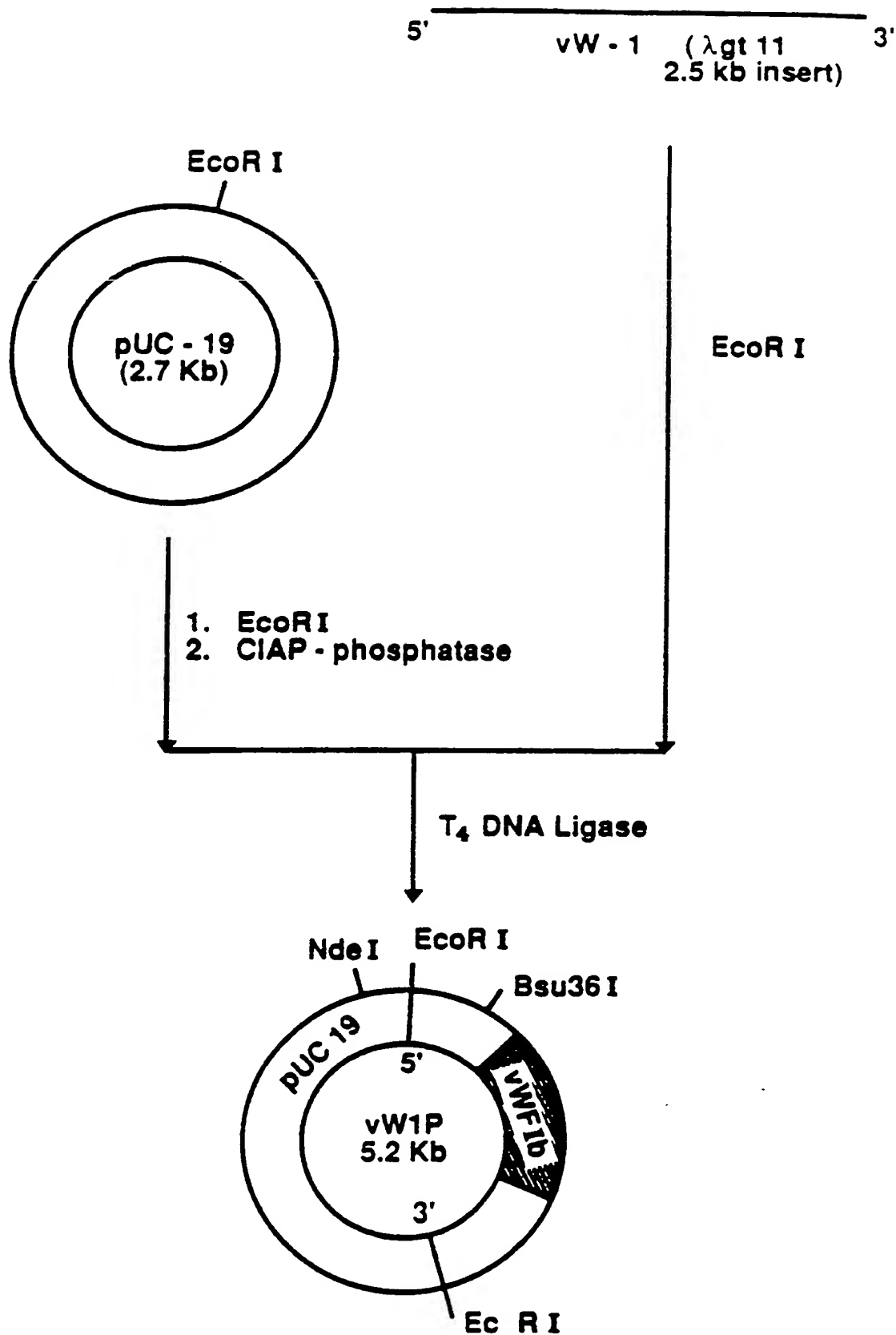
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Figure 2

SYNTHETIC OLIGOMER

```
5' - TATGAGGTGGCTGGCCGGCGTTTTGCC - 3'
3' - ACTCCACCGACCGGCCGCAAAACGGAGT - 5'
```

NdeI   BSU 36 I

1. Nde I
2. Bsu 36 I
3. Dilute

T4 DNA Ligase

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Figure 3

SYNTHETIC OLIGOMER

5' - TATGTTTGCC - 3'
3' - ACAAACGGAGT - 5'

1. Nde I
2. Bsu 36 I
3. Dilute

T₄ DNA Ligase
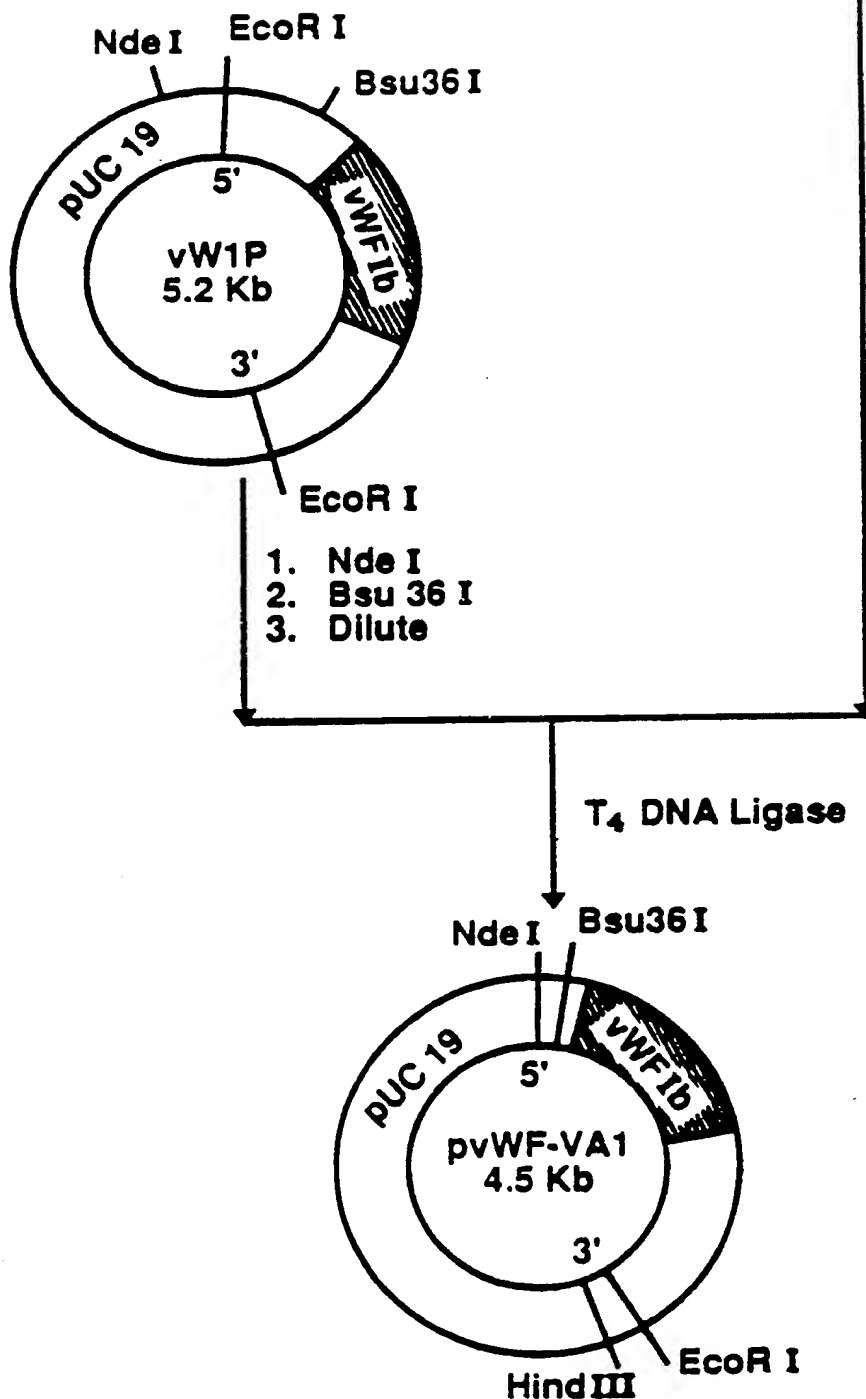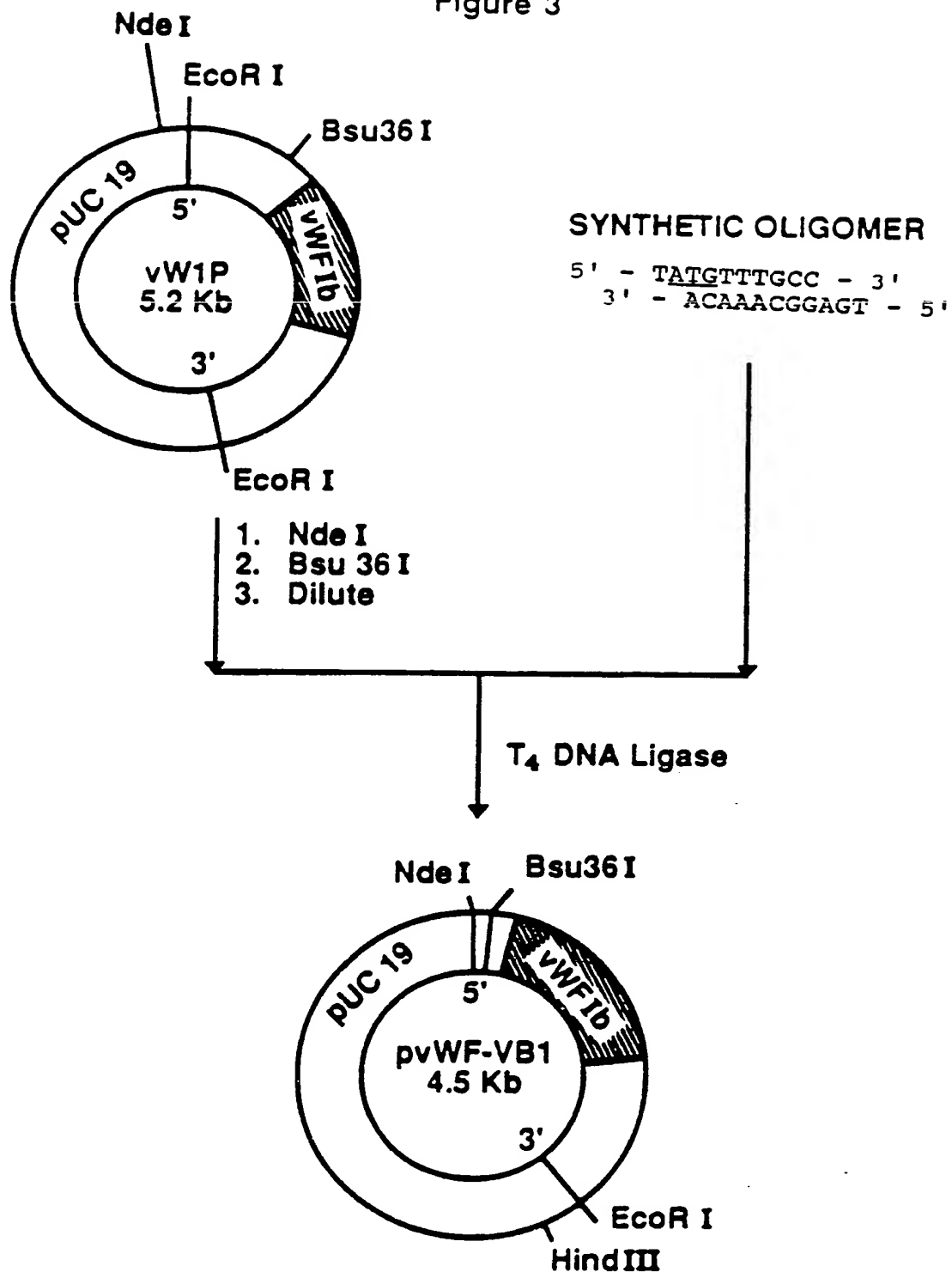
Figure 4

SYNTHETIC LINKER

```
5'- CCGGGGCTCTTGGGGGTTTCGACCCTGGGGCCCAAGTAAGATATCA - 3'
3'- CCGAGAACCCCCAAAGCTGGGACCCCGGGTTCATTCTATAGTTCGA - 5'
```

Nde I   Bsu36 I

pUC 19 / 5' / vWF1b / Xma I

pvWF-VA1
4.5 Kb

3'

Hind III   EcoR I

1. Hind III
2. Xma I
3. Dilute

T₄ DNA Ligase

Nde I   Bsu36 I

pUC 19 / 5' / vWF1b

pvWF-VA2
3.4 Kb   3'

Xma I

Hind III
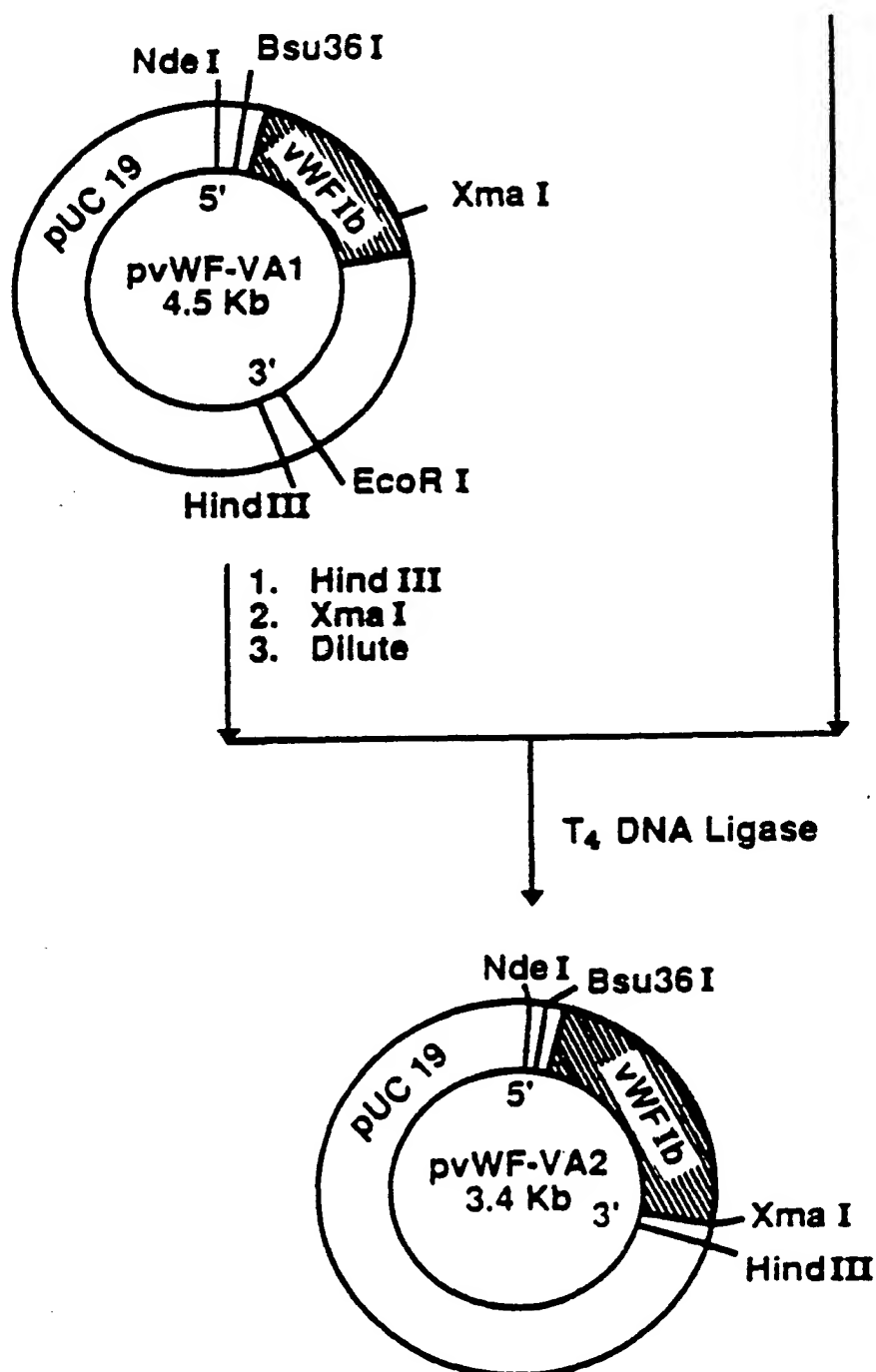
Figure 5

SYNTHETIC LINKER

5' - CCGGGGCTCTTGGGGTTTCGACCCTGGGGCCCAAGTAAGATATCA - 3'
3' - CCGAGAACCCCAAAGCTGGGACCCCGGGTTCATTCTATAGTTCGA - 5'

1. Hind III
2. Xma I
3. Dilute
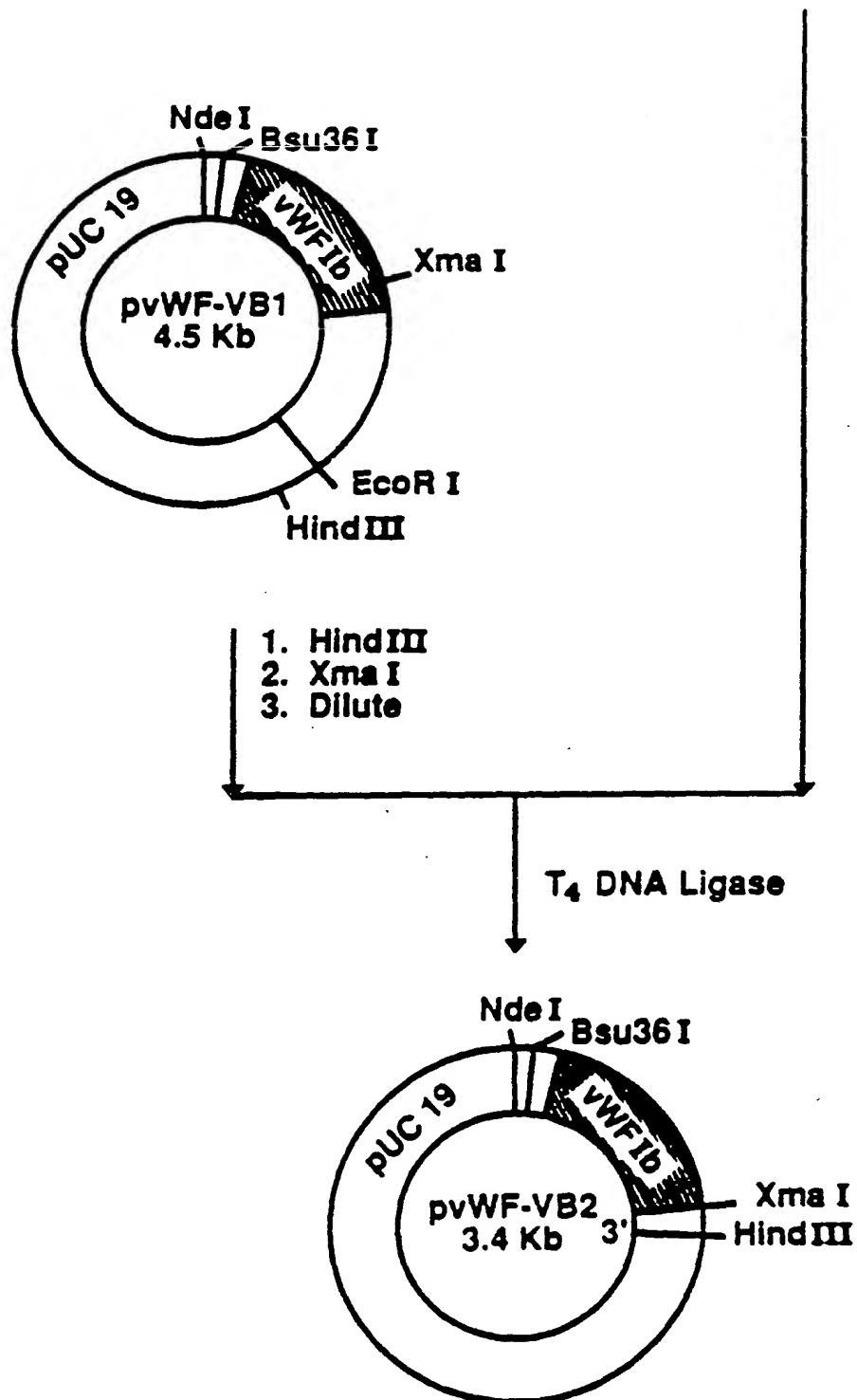
T₄ DNA Ligase

6/35
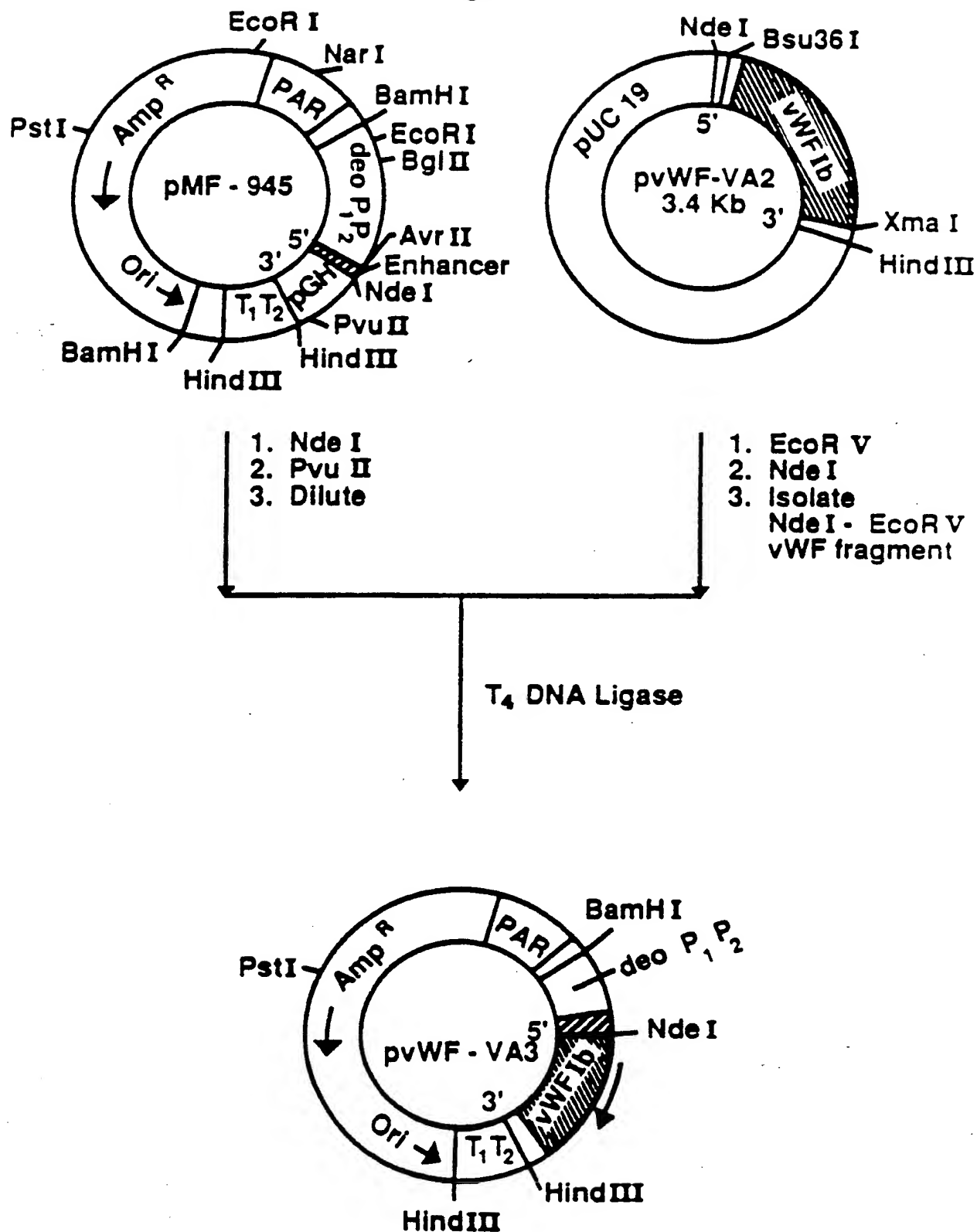
Figure 6

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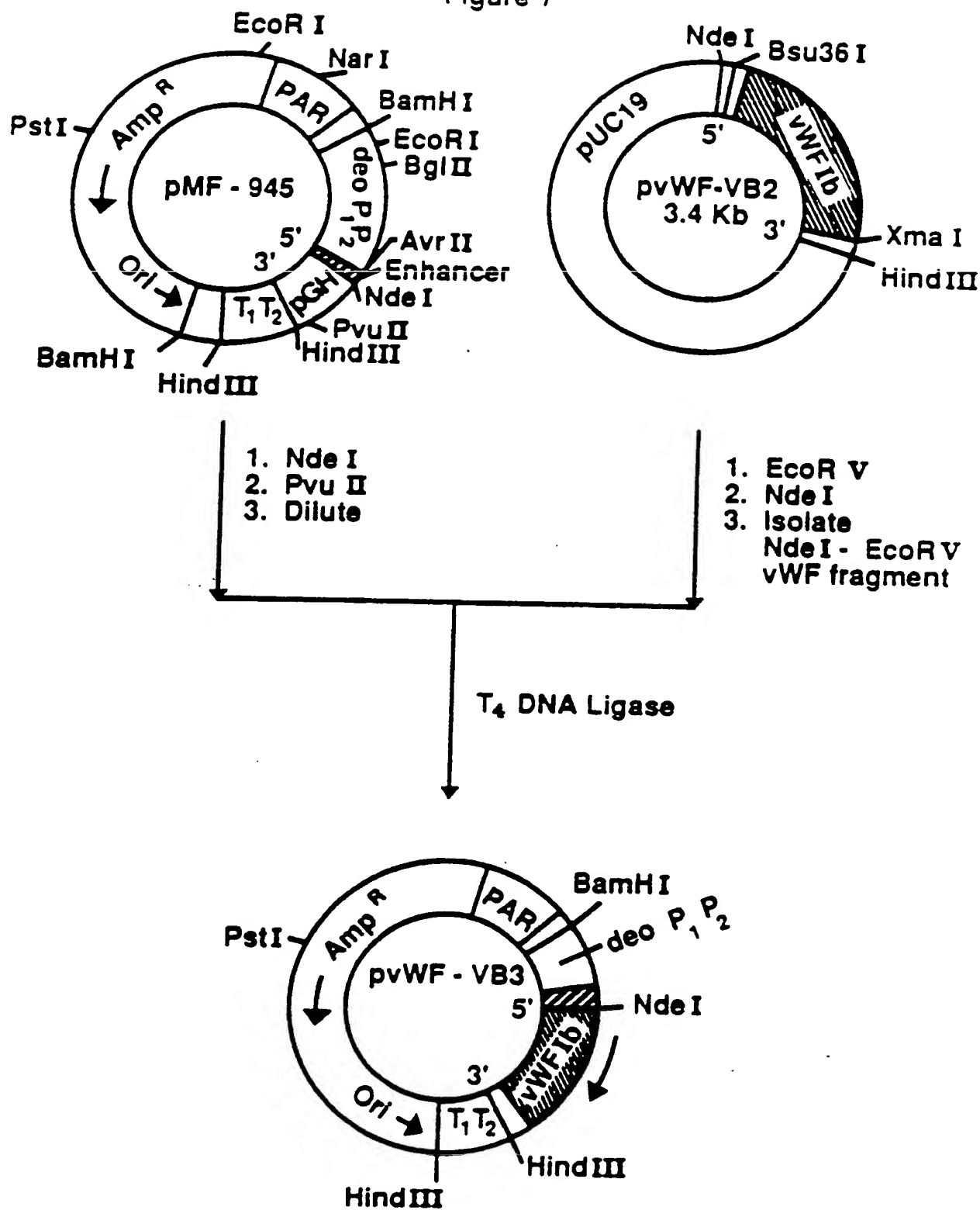
Figure 7

SYNTHETIC LINKER

5' - T<u>AT</u>GTTGCACGATTTCTACTGCAGCAGGCTACTGGACC - 3'
3' - ACAACGTGCTAAAGATGACGTCGTCCGATGACCTGGA - 5'

NdeI   Tth 111I
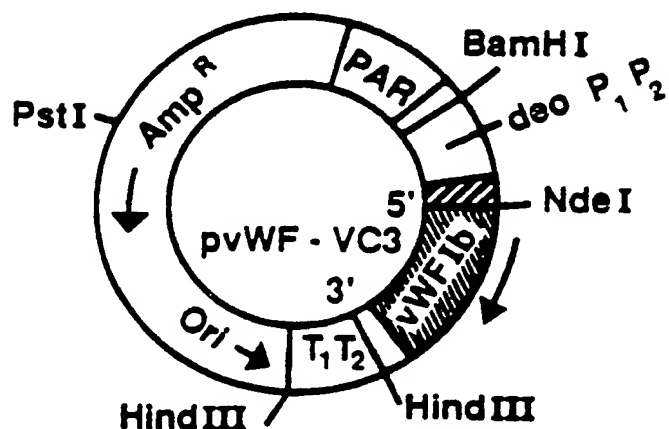
1. Nde I
2. Tth 111 I
3. Dilute

T₄ DNA Ligase

Figure 9

SYNTHETIC LINKERS

$5'$ – TAT<u>G</u>CTGGACC – $3'$
$3'$ – ACGACCTGGA – $5'$

NdeI   Tth 111I

PstI   Amp R   PAR   BamHI   deo P₁ P₂
pvWF - VA3   5'   Nde I   Tth 111 I
3'   VWFIb
Ori   T₁ T₂   Hind III
Hind III

1. Nde I
2. Tth 111 I
3. Dilute

T₄ DNA Ligase

PstI   Amp R   PAR   BamHI   deo P₁ P₂
pvWF - VD3   5'   Nde I
3'   VWFIb
Ori   T₁ T₂   Hind III
Hind III
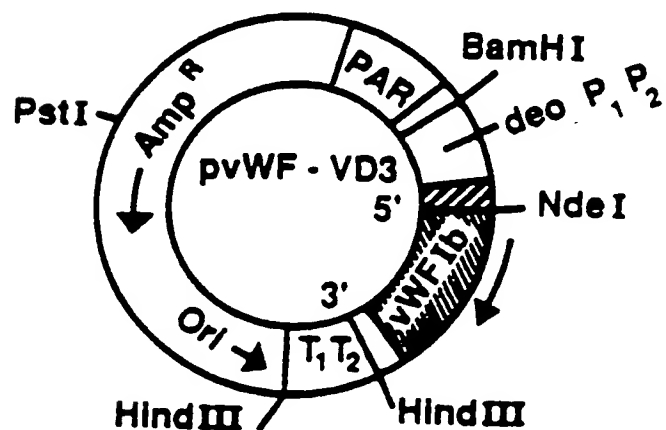
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Figure 10

Figure 11

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Figure 12A

```
1    Ser Leu Ser Cys Arg Pro Met Val Lys Leu Val Cys Pro Ala Asp      16
2519 AGC CTA TCC TGT CGG CCC ATG GTC AAG CTG GTG TGT CCC GCT GAC      2566

17   Asn Leu Arg Ala Glu Gly Leu Glu Cys Thr Lys Cys Gln Asn Tyr      32
2567 AAC CTG CGG GCT GAA GGG CTC GAG TGT ACC AAA TGC CAG AAC TAT      2614

33   Asp Leu Glu Cys Met Ser Met Gly Cys Val Ser Gly Cys Leu Cys Pro  48
2615 GAC CTG GAG TGC ATG AGC ATG GGC TGC GTC TCT GGC TGC CTC TGC CCC  2662

49   Pro Gly Met Val Arg His Glu Asn Arg Cys Val Ala Leu Glu Arg Cys  64
2663 CCG GGC ATG GTC CGG CAT GAG AAC AGA TGT GTG GCC CTG GAA AGG TGT  2710

65   Pro Cys Phe His Gln Gly Lys Glu Tyr Ala Pro Gly Glu Thr Val Lys  80
2711 CCC TGC TTC CAT CAG GGC AAG GAG TAT GCC CCT GGA GAA ACA GTG AAG  2758

81   Ile Gly Cys Asn Thr Cys Val Cys Arg Asp Arg Lys Trp Asn Cys Thr  96
2759 ATT GGC TGC AAC ACT TGT GTC TGT CGG GAC CGG AAG TGG AAC TGC ACA  2806

97   Asp His Val Cys Asp Ala Thr Cys Ser Thr Ile Gly Met Ala His Tyr  112
2807 GAC CAT GTG TGT GAT GCC ACG TGC TCC ACG ATC GGC ATG GCC CAC TAC  2854

113  Leu Thr Phe Asp Gly Leu Lys Tyr Leu Phe Pro Gly Glu Cys Gln Tyr  128
2855 CTC ACC TTC GAC GGG CTC AAA TAC CTG TTC CCC GGG GAG TGC CAG TAC  2902

129  Val Leu Val Gln Asp Tyr Cys Gly Ser Asn Pro Gly Thr Phe Arg Ile  144
2903 GTT CTG GTG CAG GAT TAC TGC GGC AGT AAC CCT GGG ACC TTT CGG ATC  2950

145  Leu Val Gly Asn Lys Gly Cys Ser His Pro Ser Val Lys Cys Lys Lys  160
2951 CTA GTG GGG AAT AAG GGA TGC AGC CAC CCC TCA GTG AAA TGC AAG AAA  2998
```

Figure 12A (continued)

```
161                                                                    176
2999  Arg Val Thr Ile Leu Val Glu Gly Gly Glu Ile Glu Leu Phe Asp Gly  3046
      CGG GTC ACC ATC CTG GTG GAG GGA GGA GAG ATT GAG CTG TTT GAC GGG

177                                                                    192
3047  Glu Val Asn Val Lys Arg Pro Met Lys Asp Glu Thr His Phe Glu Val  3094
      GAG GTG AAT GTG AAG AGG CCC ATG AAG GAT GAG ACT CAC TTT GAG GTG

193                                                                    208
3095  Val Glu Ser Gly Arg Tyr Ile Ile Leu Leu Gly Lys Ala Leu Ser      3142
      GTG GAG TCT GGC CGG TAC ATC ATT CTG CTG GGC AAA GCC CTC TCC

209                                                                    224
3143  Val Val Trp Asp Arg His Leu Ser Ile Ser Val Leu Lys Gln Thr      3190
      GTG GTC TGG GAC CGC CAC CTG AGC ATC TCC GTG CTG AAG CAG ACA

225                                                                    240
3191  Tyr Gln Glu Lys Val Cys Gly Leu Cys Gly Asn Phe Asp Gly Ile Gln  3238
      TAC CAG GAG AAA GTG TGT GGC CTG TGT GGG AAT TTT GAT GGC ATC CAG

241                                                                    256
3239  Asn Asn Asp Leu Thr Ser Ser Asn Leu Gln Val Glu Glu Asp Pro Val  3296
      AAC AAT GAC CTC ACC AGC AGC AAC CTC CAA GTG GAG GAG GAC CCT GTG
```

Figur   12B

```
257  Asp Phe Gly Lys Ser Trp Glu Val Ser Ser Gln Cys Ala Asp Thr Arg  272
3287 GAC TTT GGG AAG TCC TGG GAA GTG AGC TCG CAG TGT GCT GAC ACC AGA  3334

273  Lys Val Pro Leu Asp Ser Ser Pro Ala Thr Cys His Asn Asn Ile Met  288
3335 AAA GTG CCT CTG GAC TCA TCC GCC ACC TGC CAT AAC AAC ATC ATG      3382

289  Lys Gln Thr Met Val Asp Ser Cys Arg Ile Leu Thr Ser Asp Val      304
3383 AAG CAG ACG ATG GTG GAT TCC TGT AGA ATC CTT ACC AGT GAC GTC      3430

305  Phe Gln Asp Cys Asn Lys Leu Val Asp Pro Glu Pro Tyr Leu Asp Val  320
3431 TTC CAG GAC TGC AAC AAG CTG GTG GAC CCC GAG CCA TAT CTG GAT GTC  3478

321  Cys Ile Tyr Asp Thr Cys Ser Cys Glu Ser Ile Gly Asp Cys Ala Cys  336
3479 TGC ATT TAC GAC ACC TGC TCC TGT GAG TCC ATT GGG GAC TGC GCC TGC  3526

337  Phe Cys Asp Thr Ile Ala Ala Tyr Ala His Val Cys Ala Gln His Gly  352
3527 TTC TGC GAC ACC ATT GCC GCC TAT GCC CAC GTG TGT GCC CAG CAT GGC  3574

353  Lys Val Val Thr Arg Thr Ala Thr Leu Cys Pro Gln Ser Cys Glu      368
3575 AAG GTG GTG ACC AGG ACG GCC ACA TTG TGC CCC CAG AGC TGC GAG      3622

369  Glu Arg Asn Leu Arg Glu Asn Gly Tyr Glu Cys Glu Trp Arg Tyr Asn  384
3623 GAG AGG AAT CTC CGG GAG AAC GGG TAT GAG TGT GAG TGG CGC TAT AAC  3670

385  Ser Cys Ala Pro Ala Cys Gln Val Thr Cys Gln His Pro Glu Pro Leu  400
3671 AGC TGT GCA CCT GCC TGT CAA GTC ACG TGT CAG CAC CCT GAG CCA CTG  3718

401  Ala Cys Pro Val Gln Cys Val Glu Gly Cys His Ala His Cys Pro Pro  416
3719 GCC TGC CCT GTG CAG TGT GTG GAG GGC TGT CAT GCC CAT TGC CCT CCA  3766
```

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Figure 12B (continued)

```
417                                                                      432
3767                                                                     3814
Gly Lys Ile Leu Asp Glu Leu Leu Gln Thr Cys Val Asp Pro Glu Asp
GGC AAA ATC CTG GAT GAG CTT TTG CAG ACC TGC GTT GAC CCT GAA GAC

433                                                                      448
3815                                                                     3862
Cys Pro Val Cys Glu Val Ala Gly Arg Arg Phe Ala Ser Gly Lys Lys
TGT CCA GTG TGT GAG GTG GCT GGC CGT CGG TTT GCC TCA GGA AAG AAA

449                                                                      464
3863                                                                     3910
Val Thr Leu Asn Pro Ser Asp Pro Glu His Cys Gln Ile Cys His Cys
GTC ACC TTG AAT CCC AGT GAC CCT GAG CAC TGC CAG ATT CAC TGT

465                                                                      480
3911                                                                     3958
Asp Val Val Asn Leu Thr Cys Ala Cys Gln Glu Pro Gly Gly Leu
GAT GTT GTC AAC CTC ACC TGC GCC TGC GAA GAG CCG GGA GGC CTG

481                                                                      496
3959                                                                     4006
Val Val Pro Pro Thr Asp Ala Pro Val Ser Pro Thr Thr Leu Tyr Val
GTG GTG CCT CCG ACA GAT GCC CCC GTG AGC CCC ACC ACT CTG TAT GTG

497                                                                      512
4007                                                                     4054
Glu Asp Ile Ser Glu Pro Pro Leu His Asp Phe Tyr Cys Ser Arg Leu
GAG GAC ATC TCG GAA CCG CCG TTG CAC GAT TTC TAC TGC AGC AGG CTA

513                                                                      528
4055                                                                     4102
Leu Asp Leu Val Phe Leu Leu Asp Gly Ser Ser Arg Leu Ser Glu Ala
CTG GAC CTG GTC TTC CTG CTG GAT GGC TCC TCC AGG CTG TCC GAG GCT
```

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Figure 12C

```
529  Glu Phe Glu Val Leu Lys Ala Phe Val Val Asp Met Met Glu Arg Leu  544
     GAG TTT GAA GTG CTG AAG GCC TTT GTG GTG GAC ATG ATG GAG CGG CTG
4103                                                                  4150

545  Arg Ile Ser Gln Lys Trp Val Arg Val Ala Val Glu Tyr His Asp      560
     CGC ATC TCC CAG AAG TGG GTC CGC GTG GCC GTG GAG TAC CAC GAC
4151                                                                  4198

561  Gly Ser His Ala Tyr Ile Gly Leu Lys Asp Arg Lys Pro Ser Glu      576
     GGC TCC CAC GCC TAC ATC GGG CTC AAG GAC CGA AAG CCA TCA GAG
4199                                                                  4246

577  Leu Arg Arg Ile Ala Ser Gln Val Lys Tyr Ala Gly Ser Gln Val Ala  592
     CTG CGG CGC ATT GCC AGC CAG GTG AAG TAT GCG GGC AGC CAG GTG GCC
4247                                                                  4294

593  Ser Thr Ser Glu Val Leu Lys Tyr Thr Leu Phe Gln Ile Phe Ser Lys  608
     TCC ACC AGC GAG GTC TTG AAA TAC ACA CTG TTC CAA ATC TTC AGC AAG
4295                                                                  4342

609  Ile Asp Arg Pro Glu Ala Ser Arg Ile Ala Leu Leu Met Ala Ser      624
     ATC GAC CGC CCT GAA GCC TCC CGC ATC GCC CTG CTC ATG GCC AGC
4343                                                                  4390

625  Gln Glu Pro Gln Arg Met Ser Arg Asn Phe Val Arg Tyr Val Gln Gly  640
     CAG GAG CCC CAA CGG ATG TCC CGG AAC TTT GTC CGC TAC GTC CAG GGC
4391                                                                  4438

641  Leu Lys Lys Lys Val Ile Val Ile Pro Val Gly Ile Gly Pro His      656
     CTG AAG AAG AAG GTC ATT GTG ATC CCG GTG GGC ATT GGG CCC CAT
4439                                                                  4486

657  Ala Asn Leu Lys Gln Ile Ala Ile Glu Lys Ala Pro Glu Asn          672
     GCC AAC CTC AAG CAG ATC GCC ATC GAG AAG GCC CCT GAG AAC
4487                                                                  4534

673  Lys Ala Phe Val Leu Ser Ser Val Asp Glu Leu Glu Gln Gln Arg Asp  688
     AAG GCC TTC GTG CTG AGT AGC GTG GAT GAG CTG GAG CAA CAG AGG GAC
4535                                                                  4582
```

```
                                                                        704
                                                                        4630
689  Glu  Ile  Val  Ser  Tyr  Leu  Cys  Asp  Leu  Ala  Pro  Glu  Ala  Pro  Pro
4583 GAG  ATC  GTT  AGC  TAC  CTC  TGT  GAC  CTT  GCC  CCT  GAA  GCC  CCT  CCT

                                                                        720
                                                                        4678
705  Thr  Leu  Pro  Pro  Asp  Met  Ala  Gln  Val  Thr  Val  Gly  Pro  Gly  Leu  Leu
4631 ACT  CTG  CCC  CCC  GAC  ATG  GCA  CAA  GTC  ACT  GTG  GGC  CCG  GGG  CTC  TTG

                                                                        736
                                                                        4726
721  Gly  Val  Ser  Thr  Leu  Gly  Pro  Lys  Arg  Asn  Ser  Met  Val  Leu  Asp  Val
4679 GGG  GTT  TCG  ACC  CTG  GGG  CCC  AAG  AGG  AAC  TCC  ATG  GTT  CTG  GAT  GTG

                                                                        752
                                                                        4774
737  Ala  Phe  Val  Leu  Glu  Gly  Ser  Asp  Lys  Ile  Gly  Glu  Ala  Asp  Phe  Asn
4727 GCG  TTC  GTC  CTG  GAA  GGA  TCG  GAC  AAA  ATT  GGT  GAA  GCC  GAC  TTC  AAC

                                                                        768
                                                                        4822
753  Arg  Ser  Lys  Glu  Phe  Met  Glu  Glu  Val  Ile  Gln  Arg  Met  Asp  Val  Gly
4775 AGG  AGC  AAG  GAG  TTC  ATG  GAG  GAG  GTG  ATT  CAG  CGG  ATG  GAT  GTG  GGC

                                                                        784
                                                                        4870
769  Gln  Asp  Ser  Ile  His  Val  Thr  Val  Leu  Gln  Tyr  Ser  Tyr  Met  Val  Thr
4823 CAG  GAC  AGC  ATC  CAC  GTC  ACG  GTG  CTG  CAG  TAC  TCC  TAC  ATG  GTG  ACC

                                                                        800
                                                                        4918
785  Val  Glu  Tyr  Pro  Phe  Ser  Glu  Ala  Gln  Ser  Lys  Gly  Asp  Ile  Leu  Gln
4871 GTG  GAG  TAC  CCC  TTC  AGC  GAG  GCA  CAG  TCC  AAA  GGG  GAC  ATC  CTG  CAG
```

Figure 12D

```
801                                                              816
4919  Arg Val Arg Glu Ile Arg Tyr Gln Gly Gly Asn Arg Thr Asn Thr Gly  4966
      CGG GTG CGA GAG ATC CGC TAC CAG GGC GGC AAC AGG ACC AAC ACT GGG

817                                                              832
4967  Leu Ala Leu Arg Tyr Leu Ser Asp His Ser Phe Leu Val Ser Gln Gly  5014
      CTG GCC CTG CGG TAC CTC TCT GAC CAC AGC TTC CTG GTC AGC CAG GGT

833                                                              848
5015  Asp Arg Glu Gln Ala Pro Asn Leu Val Tyr Met Val Thr Gly Asn Pro  5062
      GAC CGG GAG CAG GCG CCC AAC CTG GTC TAC ATG GTC ACC GGA AAT CCT

849                                                              864
5063  Ala Ser Asp Glu Ile Lys Arg Leu Pro Gly Asp Ile Gln Val Val Pro  5110
      GCC TCT GAT GAG ATC AAG AGG CTG CCT GGA GAC ATC CAG GTG GTG CCC

865                                                              880
5111  Ile Gly Val Gly Pro Asn Ala Asn Val Gln Leu Glu Arg Ile Gly      5158
      ATT GGA GTG GGC CCT AAT GCC AAC GTG CAG CTG GAG AGG ATT GGC

881                                                              896
5159  Trp Pro Asn Ala Pro Ile Leu Ile Gln Asp Phe Glu Thr Leu Pro Arg  5206
      TGG CCC AAT GCC CCA ATC CTC ATC CAG GAC TTT GAG ACG CTC CCC CGA

897                                                              912
5207  Glu Ala Pro Asp Leu Val Leu Gln Arg Cys Cys Ser Gly Glu Gly Leu  5254
      GAG GCT CCT GAC CTG GTG CTG CAG AGG TGC TGC TCC GGA GAG GGG CTG

913                                                              928
5255  Gln Ile Pro Thr Leu Ser Pro Ala Pro Asp Cys Ser Gln Pro Leu Asp  5302
      CAG ATC CCC ACC CTC TCC CCA GCA CCT GAC TGC AGC CAG CCC CTG GAC

929                                                              944
5303  Val Ile Leu Leu Leu Asp Gly Ser Ser Phe Pro Ala Ser Tyr Phe      5350
      GTG ATC CTT CTC CTG GAT GGC TCC AGT TTC CCA GCT TCT TAT TTT

945                                                              960
5351  Asp Glu Met Lys Ser Phe Ala Lys Ala Phe Ile Ser Lys Ala Asn Ile  5398
      GAT GAA ATG AAG AGT TTC GCC AAG GCT TTC ATT TCA AAA GCC AAT ATA
```

```
961   Gly Pro Arg Leu Thr Gln Val Ser Val Leu Gln Tyr Gly Ser Ile Thr   976
5399  GGG CCT CGT CTC ACT CAG GTG TCA GTG CTG CAG TAT GGA AGC ATC ACC  5446

977   Thr Ile Asp Val Pro Trp Asn Val Val Pro Glu Lys Ala His Leu Leu   992
5447  ACC ATT GAC GTG CCA TGG AAC GTG GTC CCG GAG AAA GCC CAT TTG CTG  5494

993   Ser Leu Val Asp Val Met Gln Arg Glu Gly Gly Pro Ser Gln Ile Gly  1008
5495  AGC CTT GTG GAC GTC ATG CAG CGG GAG GGA GGC CCC AGC CAA ATC GGG  5542

1009  Asp Ala Leu Gly Phe Ala Val Arg Tyr Leu Thr Ser Glu Met His Gly  1024
5543  GAT GCC TTG GGC TTT GCT GTG CGA TAC TTG ACT TCA GAA ATG CAT GGT  5590

1025  Ala Arg Pro Gly Ala Ser Lys Ala Val Val Ile Leu Val Thr Asp Val  1040
5591  GCC AGG CCG GGA GCC TCA AAG GCG GTG GTC ATC CTG GTC ACG GAC GTC  5638

1041  Ser Val Asp Ser Val Asp Ala Ala Ala Asp Ala Arg Ser Asn Arg      1056
5639  TCT GTG GAT TCA GTG GAT GCA GCT GCC GAT GCC AGG TCC AAC AGA      5686

1057  Val Thr Val Phe Pro Ile Gly Ile Gly Asp Arg Tyr Asp Ala Ala Gln  1072
5687  GTG ACA GTG TTC CCT ATT GGA ATT GGA GAT CGC TAC GAT GCA GCC CAG  5734
```

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Figure 12E

```
1073                                                              1088
5735                                                              5782
Leu Arg Ile Leu Ala Gly Pro Ala Gly Asp Ser Asn Val Val Lys Leu
CTA CGG ATC TTG GCA GGC CCA GCA GGC GAC TCC AAC GTG GTG AAG CTC

1089                                                              1104
5783                                                              5830
Gln Arg Ile Glu Asp Leu Pro Thr Met Val Thr Leu Gly Asn Ser Phe
CAG CGA ATC GAA GAC CTC CCT ACC ATG GTC ACC TTG GGC AAT TCC TTC

1105                                                              1120
5831                                                              5878
Leu His Lys Leu Cys Ser Gly Phe Val Arg Ile Cys Met Asp Glu Asp
CTC CAC AAA CTG TGC TCT GGA TTT GTT AGG ATT TGC ATG GAT GAG GAT

1121                                                              1136
5879                                                              5926
Gly Asn Glu Lys Arg Pro Gly Asp Val Trp Thr Leu Pro Asp Gln Cys
GGG AAT GAG AAG AGG CCC GGG GAC GTC TGG ACC TTG CCA GAC CAG TGC

1137                                                              1152
5927                                                              5974
His Thr Val Thr Cys Gln Pro Asp Gly Gln Thr Leu Lys Ser His
CAC ACC GTG ACT TGC CAG CCA GAT GGC CAG ACC CTG AAG AGT CAT

1153                                                              116
5975                                                              602
Arg Val Asn Cys Asp Arg Gly Leu Arg Pro Ser Cys Pro Asn Ser Gln
CGG GTC AAC TGT GAC CGG CTG AGG CCT TCG TGC CCT AAC AGC CAG

1169                                                              1184
6023                                                              6070
Ser Pro Val Lys Val Glu Glu Thr Cys Gly Cys Arg Trp Thr Cys Pro
TCC CCT GTT AAA GTG GAA GAG ACC TGT GGC TGC CGC TGG ACC TGC CCC

1185                                                              1200
6071                                                              6118
Cys Val Cys Thr Gly Ser Ser Thr Arg His Ile Val Thr Phe Asp Gly
TGC GTG TGC ACA GGC AGC AGC ACT CGG CAC ATC GTG ACC TTT GAT GGG

1201                                                              1216
6119                                                              6166
Gln Asn Phe Lys Leu Thr Gly Ser Cys Ser Tyr Val Leu Phe Gln Asn
CAG AAT TTC AAG CTG ACT GGC AGC TGT TCT TAT GTC CTA TTT CAA AAC

1217                                                              1232
6167                                                              6214
Lys Glu Gln Asp Leu Glu Val Ile Leu His Asn Gly Ala Cys Ser Pro
AAG GAG CAG GAC CTG GAG GTG ATT CTC CAT AAT GGT GCC TGC AGC CCT
```

```
1233                                                             1248
6215                                                             6262
Gly Ala Arg Gln Gly Cys Met Lys Ser Ile Glu Val Lys His Ser Ala
GGA GCA AGG CAG GGC TGC ATG AAA TCC ATC GAG GTG AAG CAC AGT GCC

1249                                                             1264
6263                                                             6310
Leu Ser Val Glu Leu His Ser Asp Met Glu Val Thr Val Asn Gly Arg
CTC TCC GTC GAG CTG CAC AGT GAC ATG GAG GTG ACG GTG AAT GGG AGA

1265                                                             1280
6311                                                             6358
Leu Val Ser Val Pro Tyr Val Gly Gly Asn Met Glu Val Asn Val Tyr
CTG GTC TCT GTT CCT TAC GTG GGT GGG AAC ATG GAA GTC AAC GTT TAT

1281                                                             1296
6359                                                             6406
Gly Ala Ile Met His Glu Val Arg Phe Asn His Leu Gly His Ile Phe
GGT GCC ATC ATG CAT GAG GTC AGA TTC AAT CAC CTT GGT CAC ATC TTC

1297                                                             1312
6407                                                             6454
Thr Phe Thr Pro Gln Asn Asn Glu Phe Gln Leu Ser Pro Lys
ACA TTC ACT CCA CAA AAC AAT GAG TTC CAA CTG AGC CCC AAG

1313                                                             1328
6455                                                             6502
Thr Phe Ala Ser Lys Thr Tyr Gly Leu Cys Gly Ile Cys Asp Glu Asn
ACT TTT GCT TCA AAG ACG TAT GGT CTG TGT GGG ATC TGT GAT GAG AAC

1329                                                             1344
6503                                                             6550
Gly Ala Asn Asp Phe Met Leu Arg Asp Gly Thr Val Thr Thr Asp Trp
GGA GCC AAT GAC TTC ATG CTG AGG GAT GGT ACA GTC ACC ACA GAC TGG
```

Figure 12F

```
1345          Lys Thr Leu Val Gln Glu Trp Thr Val Gln Arg Pro Gly Gln Thr Cys   1360
6551          AAA ACA CTT GTT CAG GAA TGG ACT GTG CAG CGG CCA GGA CAG ACG TGC   6598

1361          Gln Pro Ile Leu Glu Glu Gln Cys Leu Val Pro Asp Ser Ser His Cys   1376
6599          CAG CCC ATC CTG GAG GAG CAG TGT CTT GTC CCC GAC AGC TCC CAC TGC   6646

1377          Gln Val Leu Leu Leu Pro Leu Phe Ala Glu Cys His Lys Val Leu Ala   1392
6647          CAG GTC CTC CTC CCA TTA CTG TTT GCT GAA TGC CAC AAG GTC CTG GCT   6694

1393          Pro Ala Thr Phe Tyr Ala Ile Cys Gln Gln Ser His Gln Glu           1408
6695          CCA GCC ACA TTC TAT GCC ATC TGC CAG CAG CAC AGT CAG GAG           6742

1409          Gln Val Cys Glu Val Ile Ala Ser Tyr Ala His Leu Cys Arg Thr Asn   1424
6743          CAA GTG TGT GAG GTG ATC GCC TCT TAT GCC CAC CTC TGC CGG ACC AAC   6790

1425          Gly Val Cys Val Asp Trp Arg Thr Pro Asp Phe Cys Ala Met Ser Cys   1440
6791          GGG GTC TGC GTT GAC TGG AGG ACA CCT GAT TTC TGT GCT ATG TCA TGC   6838

1441          Pro Pro Ser Leu Val Tyr Asn His Cys Glu His Gly Cys Pro Arg His   1456
6839          CCA CCA TCT CTG GTC TAC AAC CAC TGT GAG CAT GGC TGT CCC CGG CAC   6886

1457          Cys Asp Gly Asn Val Ser Ser Cys Gly Asp His Pro Ser Glu Gly Cys   1472
6887          TGT GAT GGC AAC GTC AGC AGC TGC GGG GAC CAT CCC TCC GAA GGC TGT   6934

1473          Phe Cys Pro Asp Lys Val Met Leu Glu Gly Ser Cys Val Pro Glu       1488
6935          TTC TGC CCT GAT AAA GTC ATG TTG GAA GGC AGC TGT GTC CCT GAA       6982

1489          Glu Ala Cys Thr Gln Cys Ile Gly Glu Asp Gly Val Gln His Gln Phe   1504
6983          GAG GCC TGC ACT CAG TGC ATT GGT GAG GAT GGA GTC CAG CAC CAG TTC   7030
```

Figure 12F (continued)

```
1505                                                                              1520
      Leu Glu Ala Trp Val Pro Asp His Gln Pro Cys Gln Ile Cys Thr Cys
7031  CTG GAA GCC TGG GTC CCG GAC CAC CAG CCC TGT CAG ATC TGC ACA TGC  7078

1521                                                                              1536
      Leu Ser Gly Arg Lys Val Asn Cys Thr Thr Gln Pro Cys Pro Thr Ala
7079  CTC AGC GGG CGG AAG GTC AAC TGC ACA ACG CAG CCC TGC CCC ACG GCC  7126

1537                                                                              1552
      Lys Ala Pro Thr Cys Gly Leu Cys Glu Val Ala Arg Leu Arg Gln Asn
7127  AAA GCT CCC ACG TGT GGC CTG TGT GAA GTA GCC CGC CTC CGC CAG AAT  7174

1553                                                                              1568
      Ala Asp Gln Cys Cys Pro Glu Tyr Glu Cys Val Cys Asp Pro Val Ser
7175  GCA GAC CAG TGC TGC CCC GAG TAT GAG TGT GTG TGT GAC CCA GTG AGC  7222

1569                                                                              1584
      Cys Asp Leu Pro Pro Val Pro His Cys Glu Arg Gly Leu Gln Pro Thr
7223  TGT GAC CTG CCC CCA GTG CCT CAC TGT GAA CGT GGC CTC CAG CCC ACA  7270

1585                                                                              1600
      Leu Thr Asn Pro Gly Glu Cys Arg Pro Asn Phe Thr Cys Ala Cys Arg
7271  CTG ACC AAC CCT GGC GAG TGC AGA CCC AAC TTC ACC TGC GCC TGC AGG  7318

1601                                                                              1616
      Lys Glu Glu Cys Lys Arg Val Ser Pro Pro Ser Cys Pro Pro His Arg
7319  AAG GAG GAG TGC AAA AGA GTG TCC CCA CCC TCC TGC CCC CCG CAC CGT  7366
```

Figure 12G

```
1617                                                              1632
7367  Leu Pro Thr Leu Arg Lys Thr Gln Cys Cys Asp Glu Tyr Glu Cys Ala  7414
      TTG CCC ACC CTT CGG AAG ACC CAG TGC TGT GAT GAG TAT GAG TGT GCC

1633                                                              1648
7415  Cys Asn Cys Val Asn Ser Thr Val Ser Cys Pro Leu Gly Tyr Leu Ala  7462
      TGC AAC TGT GTC AAC TCC ACA GTG AGC TGT CCC CTT GGG TAC TTG GCC

1649                                                              1664
7463  Ser Thr Ala Thr Asn Asp Cys Gly Cys Thr Thr Thr Cys Leu Pro      7510
      TCA ACC GCC ACC AAT GAC TGT GGC TGT ACC ACA ACC TGC CTT CCC

1665                                                              1680
7511  Asp Lys Val His Arg Ser Thr Ile Tyr Pro Val Gly Gln Phe          7558
      GAC AAG GTG CAC CGA AGC ACC ATC TAC CCT GTG GGC CAG TTC

1681                                                              1696
7559  Trp Glu Gly Cys Asp Val Cys Thr Cys Thr Asp Met Glu Asp Ala      7606
      TGG GAG GAG TGC GAT GTG TGC ACC TGC ACC GAC ATG GAG GAT GCC

1697                                                              1712
7607  Val Met Gly Leu Arg Val Ala Gln Cys Ser Gln Lys Pro Cys Glu Asp  7654
      GTG ATG GGC CTC CGG GTG GCC CAG TGC TCC CAG AAG CCC TGT GAG GAC

1713                                                              1728
7655  Ser Cys Arg Ser Gly Phe Thr Tyr Val Leu His Glu Gly Glu Cys Cys  7702
      AGC TGT CGG TCG GGC TTC ACT TAC GTT CTG CAT GAA GGC GAG TGC TGT

1729                                                              1744
7703  Gly Arg Cys Leu Pro Ser Ala Cys Glu Val Val Thr Gly Ser Pro Arg  7750
      GGA AGG TGC CTG CCA TCT GCC TGT GAG GTG GTG ACT GGC TCA CCG CGG

1745                                                              1760
7751  Gly Asp Ser Gln Ser Ser Trp Lys Ser Val Gly Ser Gln Trp Ala Ser  7798
      GGG GAC TCC CAG TCC TGG AAG AGT TCC AAG GTC GGC TCC CAG TGG GCC TCC

1761                                                              1776
7799  Pro Glu Asn Pro Cys Leu Ile Asn Glu Cys Val Arg Val Lys Glu Glu  7846
      CCG GAG AAC CCC TGC CTC ATC AAT GAG TGT GTC CGA GTG AAG GAG GAG
```

Figure 12G (continued)

```
1777                                                                      1792
7847  Val Phe Ile Gln Gln Arg Asn Val Ser Cys Pro Gln Leu Glu Val Pro     7894
      GTC TTT ATA CAA CAA AGG AAC GTC TCC TGC CCC CAG CTG GAG GTC CCT

1793                                                                      1808
7895  Val Cys Pro Ser Gly Phe Gln Leu Ser Cys Lys Thr Ser Ala Cys Cys     7942
      GTC TGC CCC TCG GGC TTT CAG CTG AGC TGT AAG ACC TCA GCG TGC TGC

1809                                                                      1824
7943  Pro Ser Cys Arg Cys Glu Arg Met Glu Ala Cys Met Leu Asn Gly Thr     7990
      CCA AGC TGT CGC TGC GAG CGC ATG GCC TGC ATG CTC AAT GGC ACT

1825                                                                      1840
7991  Val Ile Gly Pro Gly Lys Thr Val Met Ile Asp Val Cys Thr Thr Cys     8038
      GTC ATT GGG CCC GGG AAG ACT GTG ATG ATC GAT GTG TGC ACG ACC TGC

1841                                                                      1856
8039  Arg Cys Met Val Gln Val Gly Val Ile Ser Gly Phe Lys Leu Glu Cys     8086
      CGC TGC ATG GTG CAG GTG GGG GTC ATC TCT GGA TTC AAG CTG GAG TGC

1857                                                                      1872
8087  Arg Lys Thr Thr Cys Asn Pro Cys Pro Leu Gly Tyr Lys Glu Glu Asn     8134
      AGG AAG ACC ACC TGC AAC CCC TGC CCC CTG GGT TAC AAG GAA GAA AAT

1873                                                                      1888
8135  Asn Thr Gly Glu Cys Cys Gly Arg Cys Leu Pro Thr Ala Cys Thr Ile     8182
      AAC ACA GGT GAA TGT TGT GGG AGA TGT TTG CCT ACG GCT TGC ACC ATT
```

```
1889        Gln Leu Arg Gly Gly Gln Ile Met Thr Leu Lys Arg Asp Glu Thr Leu  1904
8183        CAG CTA AGA GGA GGA CAG ATC ATG ACA CTG AAG CGT GAT GAG ACG CTC  8230

1905        Gln Asp Gly Cys Asp Thr His Phe Cys Lys Val Asn Glu Arg Gly Glu  1920
8231        CAG GAT GGC TGT GAT ACT CAC TTC TGC AAG GTC AAT GAG AGA GGA GAG  8278

1921        Tyr Phe Trp Glu Lys Arg Val Thr Gly Cys Pro Pro Phe Asp Glu His  1936
8279        TAC TTC TGG GAG AAG AGG GTC ACA GGC TGC CCA CCC TTT GAT GAA CAC  8326

1937        Lys Cys Leu Ala Glu Gly Gly Lys Ile Met Lys Ile Pro Gly Thr Cys  1952
8327        AAG TGT CTG GCT GAG GGA GGT AAA ATT ATG AAA ATT CCA GGC ACC TGC  8374

1953        Cys Asp Thr Cys Glu Glu Pro Glu Cys Asn Asp Ile Thr Ala Arg Leu  1968
8375        TGT GAC ACA TGT GAG GAG CCT GAG TGC AAC GAC ATC ACT GCC AGG CTG  8422

1969        Gln Tyr Val Lys Val Gly Ser Cys Lys Ser Glu Val Glu Val Asp Ile  1984
8423        CAG TAT GTC AAG GTG GGA AGC TGT AAG TCT GAA GTA GAG GTG GAT ATC  8470

1985        His Tyr Cys Gln Gly Lys Lys Cys Ala Ser Lys Ala Met Tyr Ser Ile Asp  2000
8471        CAC TAC TGC CAG GGC AAA AAA TGT GCC AGC AAA GCC ATG TAC TCC ATT GAC  8518

2001        Ile Asn Asp Val Gln Asp Gln Cys Cys Ser Cys Ser Pro Thr Arg Thr  2016
8519        ATC AAC GAT GTG CAG GAC CAG TGC TGC TCC TGC TCT CCG ACA CGG ACG  8566

2017        Glu Pro Met Gln Val Ala Leu His Cys Thr Asn Gly Ser Val Val Tyr  2032
8567        GAG CCC ATG CAG GTG GCC CTG CAC TGC ACC AAT GGC TCT GTT GTG TAC  8614

2033        His Glu Val Leu Asn Ala Met Glu Cys Lys Cys Ser Pro Arg Lys Cys  2048
8615        CAT GAG GTT CTC AAT GCC ATG GAG TGC AAA TGC TCC CCC AGG AAG TGC  8662

2049        Ser Lys ***
8663        AGC AAG TGA
```

Figure 13

```
  1   Met Leu His Asp Phe Tyr Cys Ser Arg Leu Leu Asp Leu Val Phe Leu    16
  1   ATG TTG CAC GAT TTC TAC TGC AGC AGG CTA CTG GAC CTG GTC TTC CTG    48

 17   Leu Asp Gly Ser Ser Arg Leu Ser Glu Ala Glu Phe Glu Val Leu Lys    32
 49   CTG GAT GGC TCC TCC AGG CTG TCC GAG GCT GAG TTT GAG GTG CTG AAG    96

 33   Ala Phe Val Val Asp Met Met Glu Arg Ile Ser         Gln Lys Trp    48
 97   GCC TTT GTG GTG GAC ATG ATG GAG CGG ATC TCC         CAG AAG TGG   144

 49   Val Arg Val Ala Val Val Glu Tyr His Asp Gly Ser His Ala Tyr Ile    64
145   GTC CGC GTG GCC GTG GTG GAG TAC CAC GAC GGC TCC CAC GCC TAC ATC   192

 65   Gly Leu Lys Asp Arg Lys Arg Pro Ser Glu Leu Glu Arg Ile Ala Ser    80
193   GGG CTC AAG GAC CGG AAG CGG CCA TCA GAG CTG GAG CGG ATT GCC AGC   240

 81   Gln Val Lys Tyr Ala Gly Ser Gln Val Ala Ser Thr Ser Glu Val Leu    96
241   CAG GTG AAG TAT GCG GGC AGC CAG GTG GCC TCC ACC AGC GAG GTC TTG   288

 97   Lys Tyr Thr Leu Phe Gln Ile Phe Ser Lys Ile Asp Arg Pro Glu Ala   112
289   AAA TAC ACA CTG TTC CAA ATC TTC AGC AAG ATC GAC CGC CCT GAA GCC   336

113   Ser Arg Ile Ala Leu Leu Leu Met Ala Ser Gln Glu Pro Gln Arg Met   128
337   TCC CGC ATC GCC CTC CTG CTG ATG GCC AGC CAG GAG CCC CAA CGG ATG   384

129   Ser Arg Asn Phe Val Arg Tyr Val Gln Gly Leu Lys Lys Lys Lys Val   144
385   TCC CGG AAC TTT GTC CGC TAC GTC CAG GGC CTG AAG AAG AAG AAG GTC   432

145   Ile Val Ile Pro Val Gly Ile Gly Pro His Ala Asn Leu Lys Gln Ile   160
433   ATT GTG ATC CCG GTG GGC ATT GGG CCC CAT GCC AAC CTC AAG CAG ATC   480
```

Figure 13 (continued)

```
161   Arg  Leu  Ile  Glu  Lys  Gln  Ala  Pro  Glu  Asn  Lys  Ala  Phe  Val  Leu  Ser   176
481   CGC  CTC  ATC  GAG  AAG  CAG  GCC  CCT  GAG  AAC  AAG  GCC  TTC  GTG  CTG  ACC   528

177   Ser  Val  Asp  Glu  Leu  Glu  Gln  Gln  Arg  Asp  Glu  Ile  Val  Ser  Tyr  Leu   192
529   AGT  GTG  GAT  GAG  CTG  GAG  CAG  CAA  AGG  GAC  GAG  ATC  GTT  AGC  TAC  CTC   576

193   Cys  Asp  Leu  Ala  Pro  Glu  Ala  Pro  Pro  Thr  Leu  Pro  Pro  Asp  Met        208
577   TGT  GAC  CTT  GCC  CCT  GAA  GCC  CCT  CCT  ACT  CTG  CCC  CCC  GAC  ATG        624

209   Ala  Gln  Val  Thr  Val  Gly  Pro  Gly  Leu  Leu  Gly  Val  Ser  Thr  Leu  Gly   224
625   GCA  CAA  GTC  ACT  GTG  GGC  CCG  GGG  CTC  TTG  GGG  GTT  TCG  ACC  CTG  GGG   672

225   Pro  Lys  ***
673   CCC  AAG  TAA
```
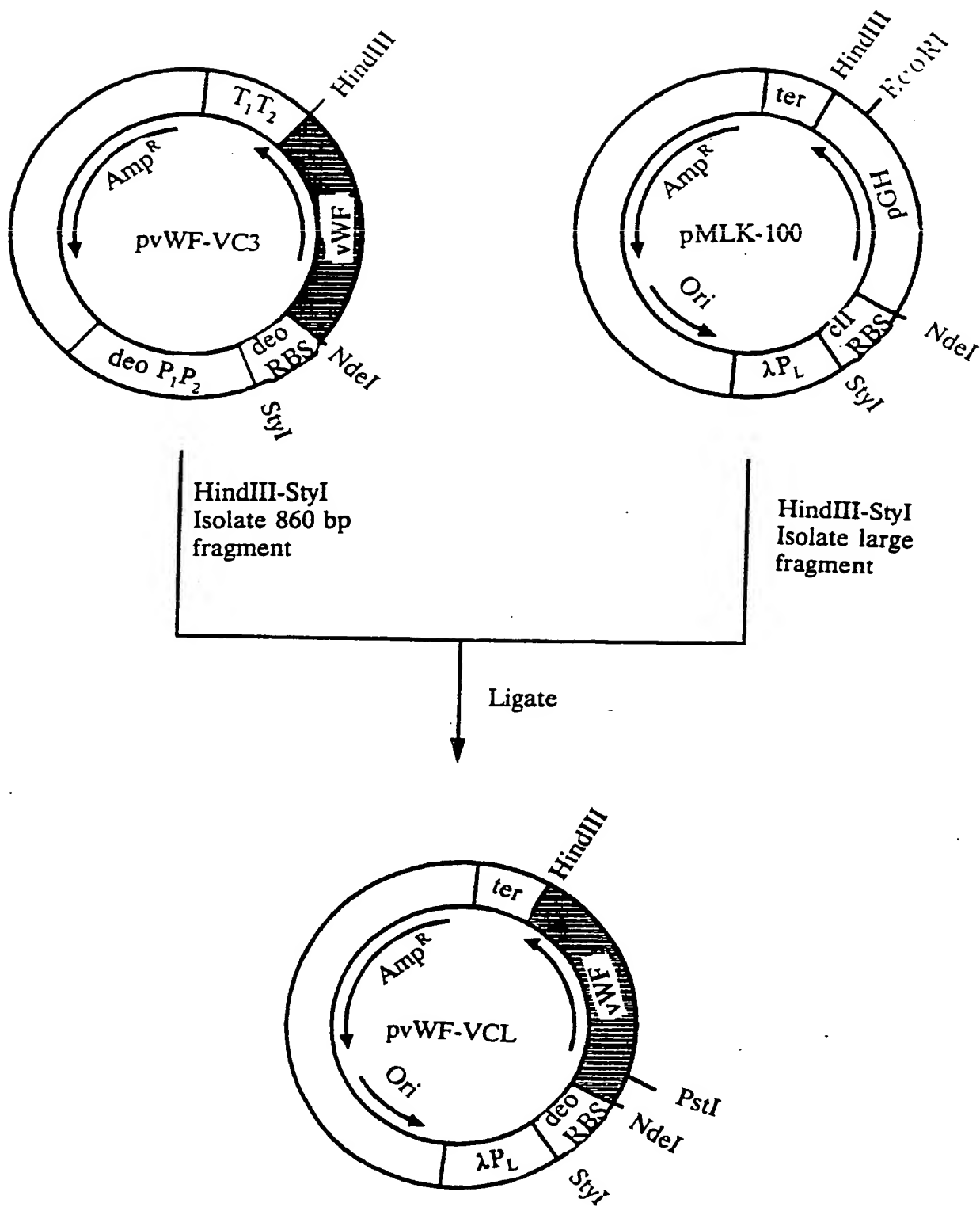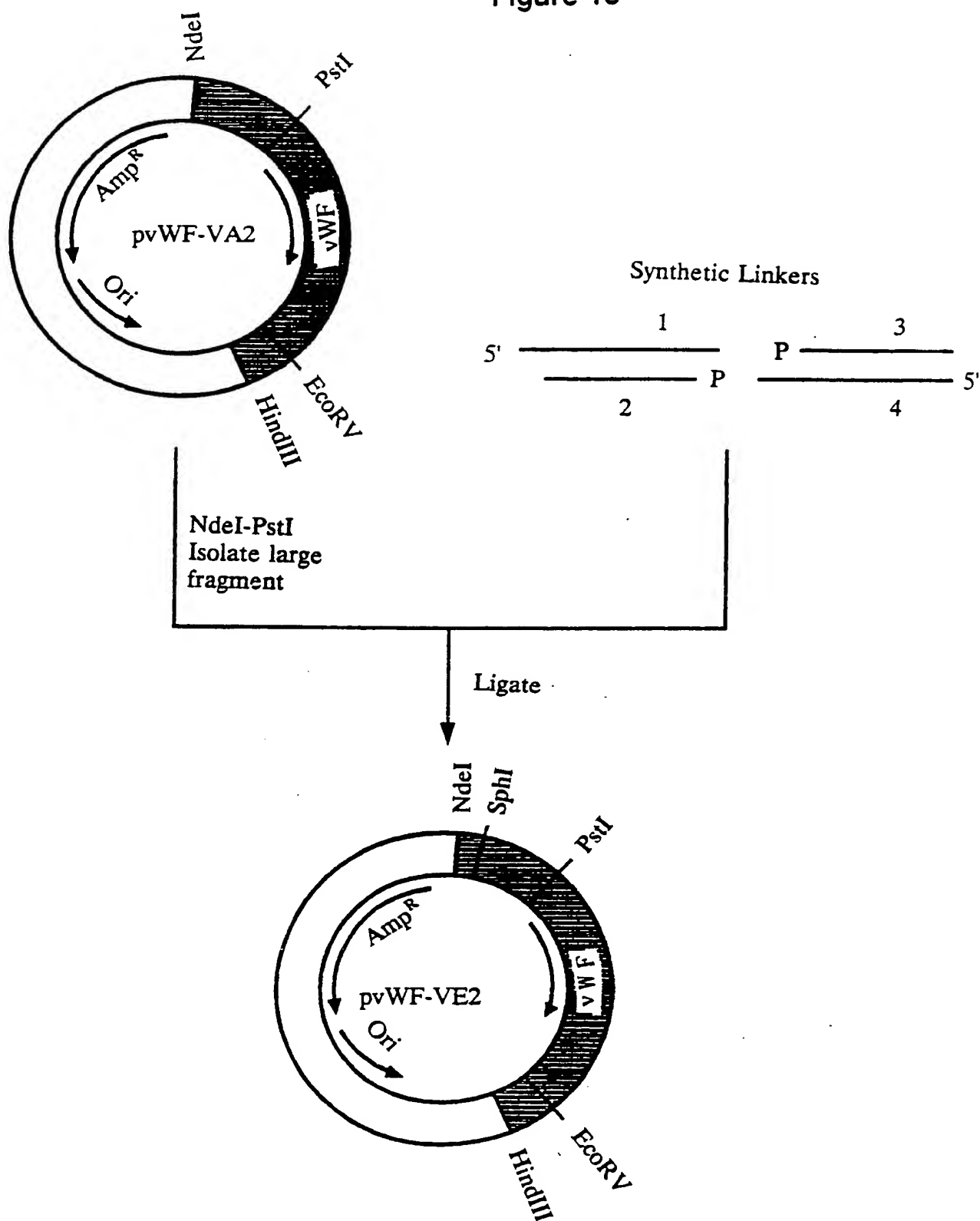
Figure 15

Figure 16

Linker

No. 1   5'   TATGCTCACCTGTGTGAAGCATGCCAGGAGCCGGGAGGCCTGGTGGTGCCTCCCACAGATGCCCCGGTG

No. 2   3'   ACGAGTGGACACTTCGTACGGTCCTCGGCCCTCCGGACCACCACGGAGGGTGTCTACGGGG

No. 3   5'   AGCCCCACCACTCTGTATGTGGAGGACATCTCGGAACCGCCGTTGCACGATTTCTACTGCA

No. 4   3'   CCACTCGGGGTGGTGAGACATACACCTCCTGTAGAGCCTTGGCGGCAACGTGCTAAAGATG
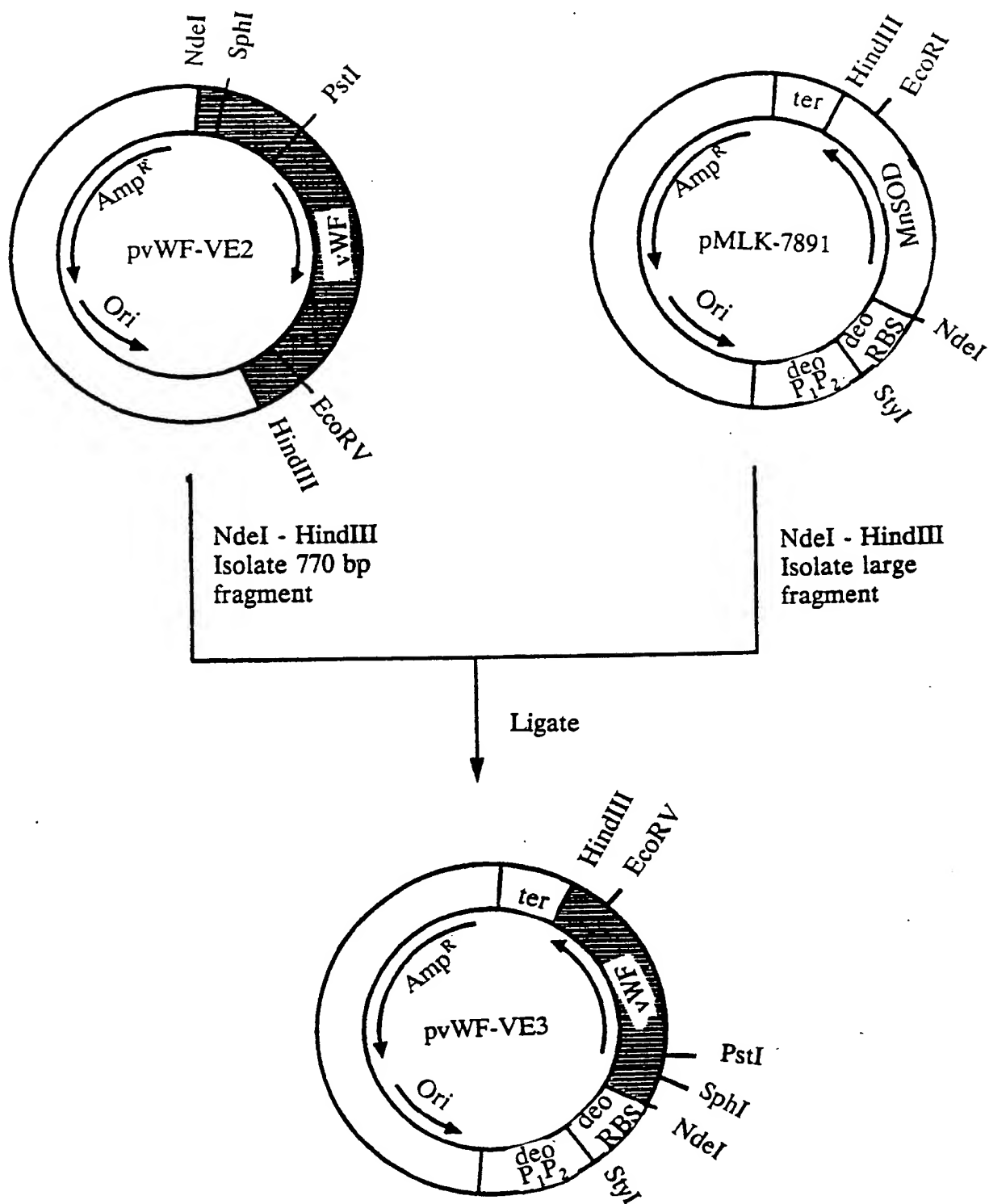
Figure 17
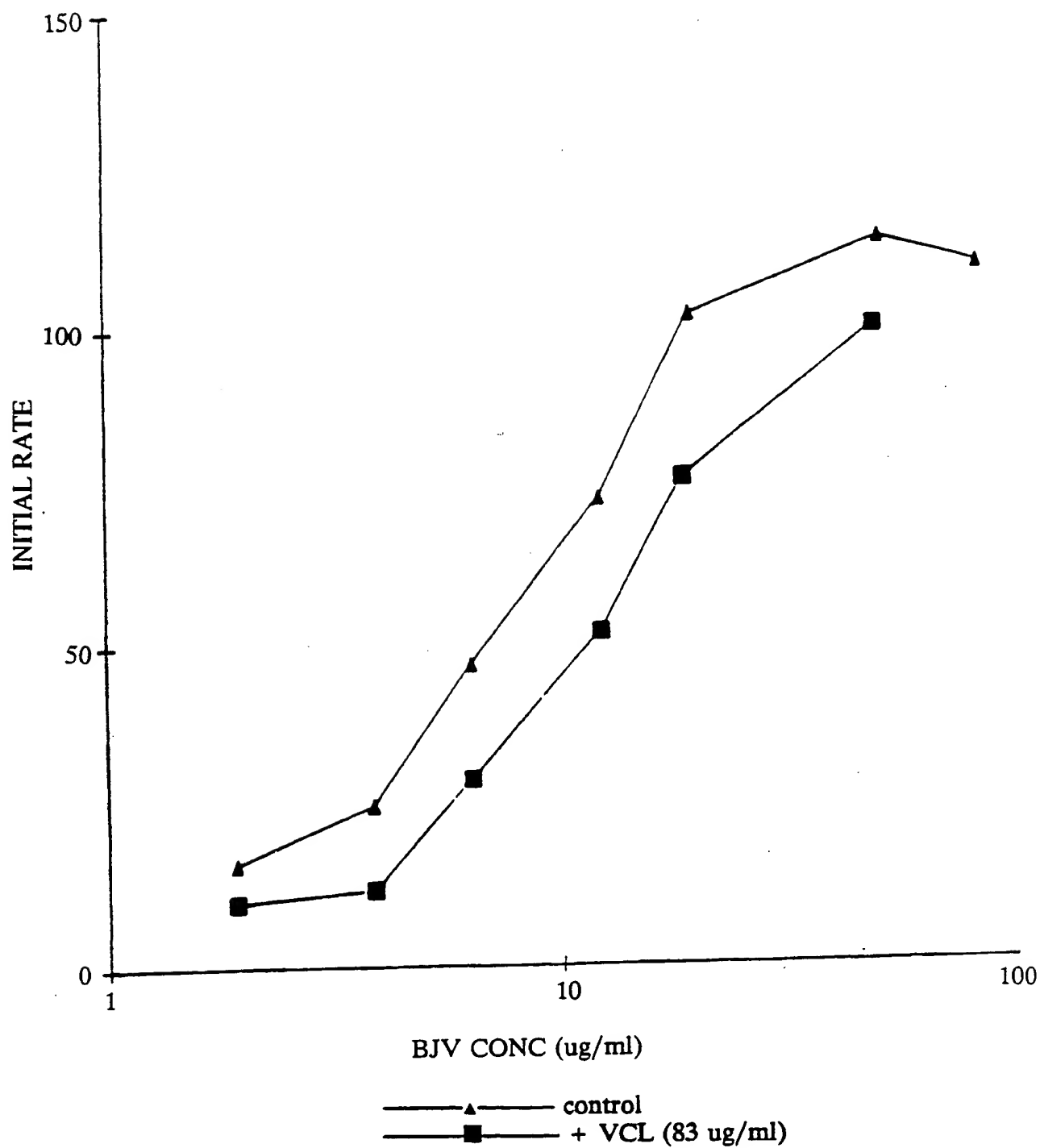

pvWF-VE2

NdeI - HindIII
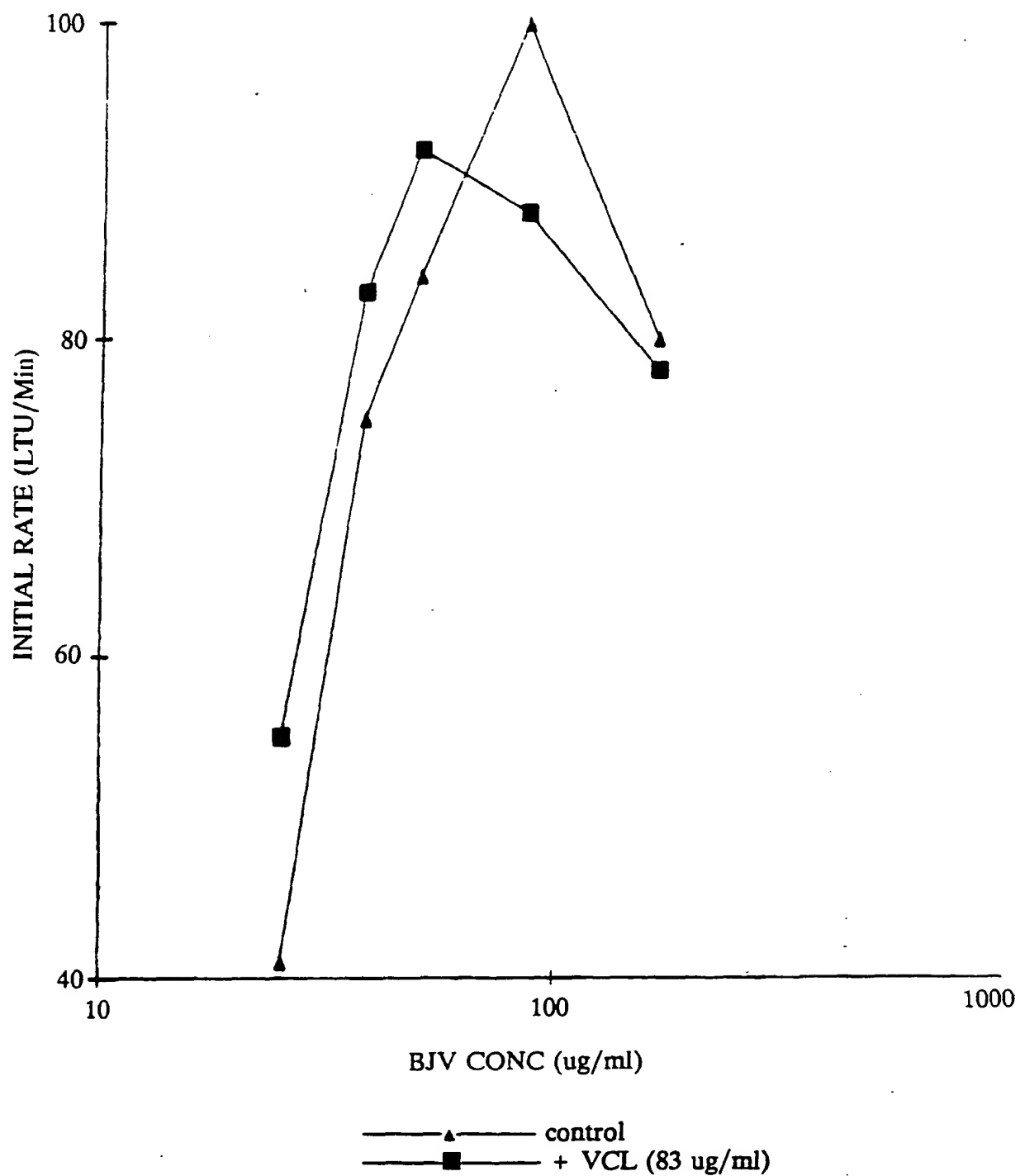Isolate 770 bp
fragment

pMLK-7891

NdeI - HindIII
Isolate large
fragment

Ligate

pvWF-VE3

Figure 18

Figure 19

BJV CONC (ug/ml)

▲ control
■ + VCL (83 ug/ml)
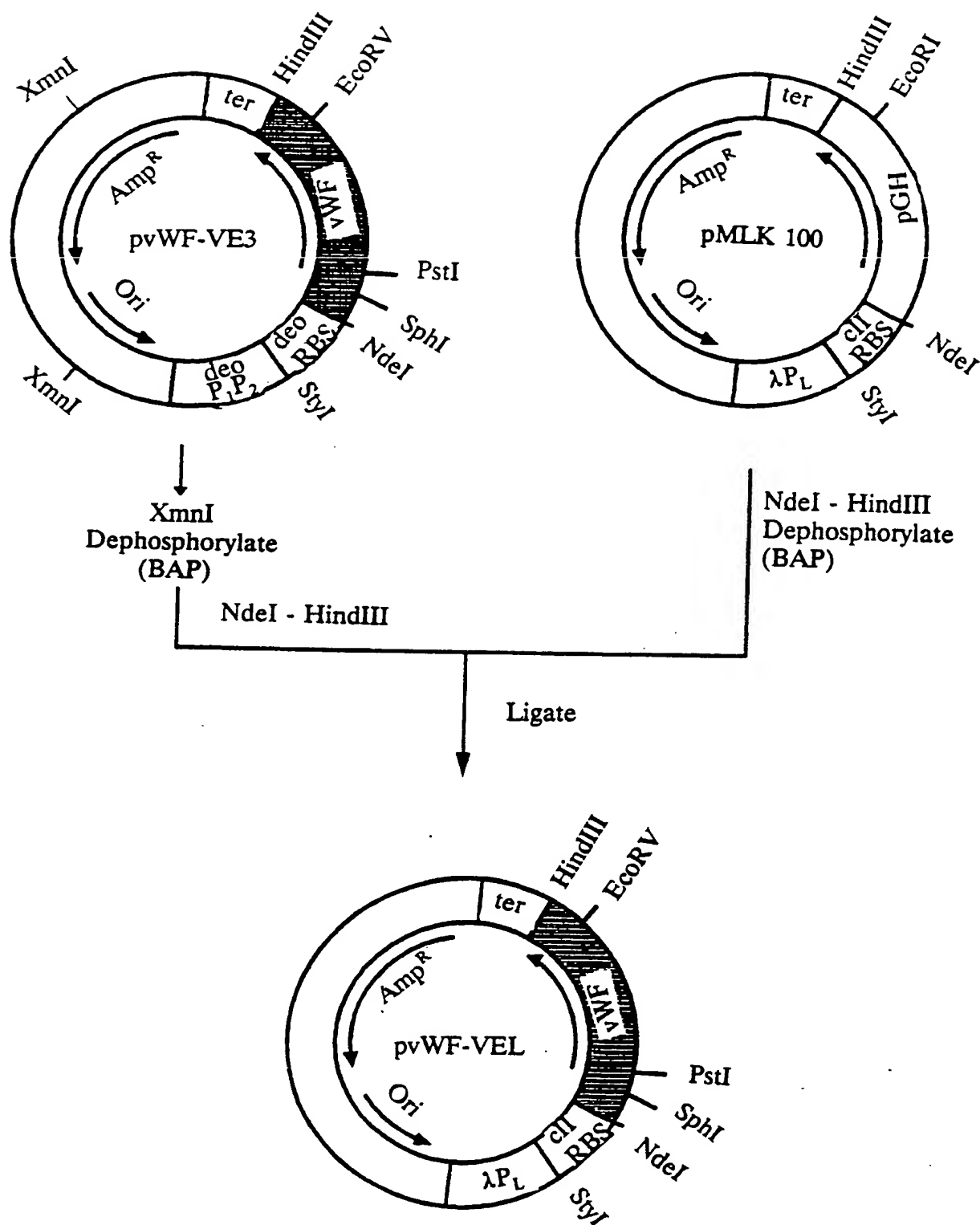
LTU = Light transmission units

Figure 20

LTU = Light transmission units

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C07K 13/00; A61K 37/00; C12N 1/21, 15/14, 15/70
US, CL,: 530/381, 383; 514/12; 435/69.6, 252.3, 320.1

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
US.CL.	530/381, 383; 514/12; 435/69.6, 252.3, 320.1

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 8

Databases: Automated Patent Searching (1975-1991)
Dialog (Files: World Patents Index, CAS, Biosis, Medline)

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category *	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No. 13
Y	Biochemical and Biophysical Research Communications, Volume 164, No. 3, issued 15 November 1989, Peitu et al., "Production in Escherichia Coli of a Biologically Active Subfragment of Von Willebrand Factor Corresponding to the Platelet Glycoprotein Ib, Collagen and Heparin Binding Domains," pages 1339-1347, see entire document.	1-11,16
Y	The Journal of Biological Chemistry, Volume 263, No. 34, issued 05 December 1988, Mohri et al., "Structure of the Von Willebrand Factor Domain Interacting with Glycoprotein Ibs" pages 17901-17904, see entire document.	1-11,16

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
30 May 1991	2 4 JUN 1991
International Searching Authority	Signature of Authorized Officer
ISA/US	R. Keith Baker, Ph.D.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category*	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	The Journal of Biological Chemistry, Volume 261, No. 32, issued 15 November 1986, Pareti et al., "Isolation and Characterization of a Collagen Binding Domain in Human Von Willebrand Factor", pages 15310-15315, see the Abstract.	1-11,16
Y	The Journal of Biological Chemistry, Volume 262, No. 4 issued 05 February 1987, Fujimura et al., "A Heparin-binding Domain of Human von Willebrand Factor" pages 1734-1739, see the entire document.	1-11,16

FURTHER INFORMA**⁻** **◄** CONTINUED FROM THE SECOND SHEET

V.☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1.☐ Claim numbers　　　　 . because they relate to subject matter [1][2] not required to be searched by this Authority, namely:

2.☐ Claim numbers　　 . because they relate to parts of the international application that do not comply with the prescribed require-
ments to such an extent that no meaningful international search can be carried out [3], specifically:

3.☐ Claim numbers＿＿＿＿＿＿, because they are dependent claims not drafted in accordance with the second and third sentences of
PCT Rule 6.4(a).

VI.☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

　　See Attachment

1.☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims
of the international application.

2.☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only
those claims of the international application for which fees were paid, specifically claims:

3.☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to
the invention first mentioned in the claims; it is covered by claim numbers:

　　　　1-11 and 16

4.☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not
invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Attachment to PCT/ISA/210

GROUP I. Claims 1-11 and 16 are drawn to a first product, a first method of making the product and a first method of using the product comprising the non-glycosylated polypeptide, a recombinant method of expression and a method of treating

5   platelet aggregation, classified in Classes 530, 435 and 514, subclasses 381, 69.6 and 12. Group II, claim 12 is drawn to a second method of use comprising treatment of cerebrovascular disorders, classified in Class 514, subclass 12. Group III, claims 13-15 is drawn to a third method of use comprising

10   treatment of cardiovascular disorders, classified in Class 514, subclass 12. Group IV, claim 17 is drawn to fourth method of use comprising treatment for blood vessel patency, classified in Class 514, subclass 12, Group V, claim 18, is drawn to a fifth method of use comprising treatment of cancer, classified in Class

15   514, subclass 12. Group VI, claims 19, 26 and 30, is drawn to a sixth method of use comprising treatment for thrombus inhibition, classified in Class 514, subclass 12. Group VII, claims 21-22, is drawn to a seventh method of use comprising treatment of platelet adhesion, classified in Class 514, subclass 12. Group

20   VIII, claim 23 is drawn to a eighth method of use comprising treatment of re-occlusion, classified in Class 514, subclass 12. Group IX, claim 24 is drawn to a ninth method of use comprising treatment of vaso-occlusive crises, classified in Class 514, subclass 12. Group X, claim 25 is drawn to a tenth method of use

25   comprising treatment of arteriosclerosis, classified in Class 514, subclass 12, Group XI, claims 27-28, is drawn to an eleventh method of use comprising treatment of platelets

activation, classified in Class 514, subclass 12. Group XII, claim 29, is drawn to a twelvth method of use comprising treatment of stenosis, classified in Class 514, subclass 12.
Group XIII, claims 31-38, is drawn to a second method of making

5   the first product, classified in class 530, subclass 381 for example. Group XIV, claim 20, is drawn to a second product comprising the polypeptide bound to a solid matrix classified in class 530, subclass 381.

       PCT Rule 13.2 permits claims to "a" (one) product and "a"

10   (one) method of making and "a" (one) method of using said product. The invention of Group I constitutes a combination of a first product, a first method of making the product and a first method of use. Groups II-XII are drawn to different in vivo methods of use of the first product in the treatment of distinct

15   disease states. Group XIII is drawn to a second and distinct method of making the first product comprising different method steps from those in Group I. Group XIV is drawn to a second and distinct product from the first product of Group I.